Determination of Endogenous Levels of Retinoic Acid Isomers in Type II Diabetes Mellitus Patients. Possible Correlation with HbA1c Values

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A sensitive HPLC method for simultaneous determination of endogenous levels of all-trans- (ATRA), 13-cis- (13cRA), and 9-cis-retinoic acids (9cRA) was applied to serum samples from healthy volunteers and type II diabetes mellitus patients. Levels of 9cRA (around 0.2 ng/ml in both groups) were below the limit of quantification. The concentrations of ATRA and 13cRA were reliably quantified, and the within-day and between-days variances indicated that they were well maintained with little variation. Concentrations of serum ATRA and 13cRA of diabetic patients (ATRA: 1.76±0.54 ng/ml; 13cRA: 1.77±0.39 ng/ml, n=13) were rather lower than those of healthy subjects (ATRA: 2.08±0.53 ng/ml; 13cRA: 2.05±0.26 ng/ml, n=18), but the differences were not significant, except for the sum of ATRA and 13cRA (p=0.033). Interestingly, the serum levels of retinoic acids in diabetic patients correlated positively with the hemoglobin A1c (HbA1c) values (ATRA: r=0.57, p<0.05; 13cRA: r=0.62, p<0.05). The results warrant further studies on the possible involvement of uncontrolled serum retinoic acids levels in the pathogenesis and/or treatment of diabetes mellitus.

Key words retinoic acid; retinoid; diabetes; hemoglobin A1c

The nutritional status of lipid and fatty acids, not only glucose, is modified in diabetes mellitus patients. The concentration of retinol, an essential dietary nutrient for normal growth, reproduction, and vision, is also affected in diabetes. Lower serum concentrations of retinol in patients with insulin-dependent diabetes (IDDM) and in streptozotocin-induced diabetic rats, in which the transport mechanism of retinol from liver to the target site is considered to be impaired, were reported. 1—5) In contrast, patients with non-insulin-dependent diabetes (NIDDM) have greater serum retinol concentrations than normal subjects. 6—8)

Recently, PPARγ, a ligand-activated transcription factor and a member of the nuclear hormone receptor superfamily, was found to play a pivotal role in the expression of genes associated with metabolism and transport of glucose and lipids. 9) Ligand-induced activation of PPARγ/ RXR heterodimer leads to differentiation of adipocytes in vitro and in vivo, and increases the insulin sensitivity of diabetic animals and human patients. Therefore, the possibility that 9-cis-retinoic acid (9cRA), an endogenous ligand of RXR and one of the metabolites of retinol, might be affected in diabetes mellitus patients and be a factor in the pathological aspects of diabetic mellitus, should be considered. All-trans-retinoic acid (ATRA), another isomer of retinoic acid (RA) and a ligand of another nuclear receptor RAR (retinoic acid receptor), has also been recognized as a potent inhibitor of adipocyte differentiation.10,11) Despite the potential importance of RA isomers, which are active metabolites of retinol, the circulating concentrations of these retinoic acids in diabetics have not been thoroughly studied. In this article, we deal with the concentrations of serum retinoic acid isomers in healthy volunteers and type II diabetes mellitus (T2DM) patients, measured by a sensitive HPLC method, and we present a correlation between serum retinoic acids level and hemoglobin A1c (HbA1c) value, an important index of diabetes, in T2DM patients.

MATERIALS AND METHODS

Chemicals ATRA was synthesized as previously described.12) 9cRA and 13-cis-retinoic acid (13cRA) were purchased from Wako Chemicals (Japan).

Methanol, acetonitrile, chloroform, ethanol, 2-propanol, n-hexane, ethyl acetate (all of HPLC grade) and ammonium acetate, diethyl ether (both grade S) and glacial acetic acid (analytical grade) were obtained from Wako Chemicals. Water for HPLC was prepared by a Milli-Q-water purification system.

Subjects Blood samples were collected from 18 healthy volunteers (average age, 32.8±7.9; BMI average, 21.2±1.7) and 13 T2DM patients (average age, 56.7±15.5; BMI average 22.7±4.3) before meals. Healthy subjects were kept off high-vitamin A diet during the study. The procedures were in accordance with local ethical standards, and informed consent was obtained from all the subjects. Blood sugar was measured with a glucose sensor after blood collection. Quantitative analysis of HbA1c, total cholesterol, and high-density lipoprotein (HDL)-cholesterol was conducted by SRL, Inc. (Japan).

Sample Preparation for HPLC Ten ml samples of whole blood from patients were collected in vacutainers containing thrombin and allowed to settle at room temperature for 1 h. They were centrifuged at 3000 rpm at 4 °C for 10 min and sera were transferred to polypropylene tubes and used immediately or stored at −20 °C.

For extraction of retinoic acids from the serum, 1 ml of serum was transferred to a 10-ml amber glass tube under Ar gas. The sample was kept under light-shielded condition throughout the following procedures. Ethanol (2 ml) was added, and the whole was vortexed and then centrifuged at 1800 rpm at ambient temperature for 5 min. The obtained supernatant was transferred to another tube containing 2 ml of 100 mM ammonium acetate (pH 4.5) under Ar gas. A mixture of n-hexane and ethyl acetate (9:1, 5 ml) was added, and the

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whole was vigorously vortexed. After centrifugation at 1800 rpm for 3 min, the aqueous phase was frozen in dry ice–acetone, then the organic phase was applied to an amino propyl column (ISOLUTE NH₂, 500 mg/3 ml, International Sorbent Technology, UK) conditioned with 2 ml of n-hexane/ethyl acetate (9:1). After stepwise washes with 2 ml of n-hexane/ethyl acetate (9:1), 2 ml of chloroform/2-propanol (2:1) twice and 1 ml of diethyl ether, absorbed retinoic acids were eluted with 2 ml of diethyl ether containing 3% acetic acid. The eluate was evaporated to dryness under a stream of Ar gas. The residue was dissolved in 50 μl of ethanol and analyzed by HPLC.

HPLC Condition HPLC analysis was performed using a Shimadzu (Japan) LC-10AS pump equipped with a UV detector (Shimadzu SPD-10A) and integrator (Shimadzu CR-8A). A COSMOSIL 4C18-AR-II column (250×4.6 mm i.d.) with 5 μm particles (Nacalai tesque, Japan) was used at ambient temperature.

A binary gradient was formed from solvent A: acetonitrile/methanol/2% acetic acid (60:16:24, v/v/v) and B: acetonitrile. These solvents were degassed by ultrasonic treatment before use. The gradient composition (solvent B percentages) of the mobile phase was: 0% (0–8 min); 0–60% (8–14 min); 60–70% (14–16 min); 70% (16–38 min). The flow rate was 0.6 ml/min. The column was flushed with 100% acetonitrile before the next injection. UV detection was carried out at 345 nm. A typical chart is shown in Fig. 1.

Quantification of RAs A standard curve for quantification of each RA isomer extracted from serum was obtained by adding defined quantities of ATRA, 13cRA (both 0.8, 2, 5 ng/ml serum), and 9cRA (0.2, 0.5, 1.25 ng/ml serum) to control serum and plotting the UV absorption area values determined by HPLC analysis of each extracted sample. A coefficient of variation (C.V.) within 10% and good linearity (R²>0.999) were obtained. The Y-slice value was subtracted from the obtained values as endogenous RAs, and the resultant standard curve was used for quantification of RAs extracted from serum. The recovery rate of extraction was estimated as about 80%. The limit of quantification (LOQ) and limit of detection (LOD) were estimated from Y-slice values (b) and standard variations (s) of subtracted standard curves (LOQ=b+10s, LOD=b+3s); ATRA: LOQ 0.27 ng/ml, LOD 0.08 ng/ml; 13cRA: LOQ 0.44 ng/ml, LOD 0.13 ng/ml; 9cRA: LOQ 0.22 ng/ml, LOD 0.07 ng/ml.

Statistical Analyses Results are expressed as the mean±standard deviation (S.D.). Within-day variance and between-days variance of data for healthy subjects were tested by repeated-measures one-way analysis of variance (ANOVA). Data sets for the T2DM patients and the normal subjects were compared using Student’s or Welch’s t test. Simple correlations between selected variables were tested by using Spearman’s rank correlation coefficient.

RESULTS

Within-Day Variances and Between-Days Variances of Serum RAs Concentrations To evaluate homeostasis of serum RAs, within-day and between-days variances of serum RAs concentrations of healthy subjects were examined. The concentration of 9cRA in serum was at a detectable level (around 0.2 ng/ml, Fig. 1), but below the limit of quantification. ATRA and 13cRA could be quantified and their levels were around 2 ng/ml. Average C.V. values of within-day and between-days variances for ATRA and 13cRA were both below 20% (Table 1). Those for between-days variances of ATRA and 13cRA were 11.0% and 8.4% respectively, being comparable to those for blood glucose (BG). All p values of within-day and between-days variances for ATRA and 13cRA obtained by repeated-measures one-way ANOVA tests were over 0.05. These results suggest that the serum concentrations of two major isomers of RA, ATRA and 13cRA, are maintained with little variation, both within a day and between days.
Serum RAs Concentrations in Type II Diabetes Mellitus Patients  Serum RAs concentrations of 13 T2DM patients were quantified and compared with those of healthy subjects (Fig. 2). Healthy subjects were kept off high-vitamin A diet during the study. There was no significant difference in the lipid indexes between healthy subjects and diabetes patients (Table 2 and data not shown). The average serum concentration of either ATRA or 13cRA was rather lower in T2DM patients (ATRA: 1.76±0.54 ng/ml; 13cRA: 1.77±0.39 ng/ml, n=13) than in healthy subjects (ATRA: 2.08±0.53 ng/ml; 13cRA: 2.05±0.26 ng/ml, n=18), though not significantly so. When the concentrations of both isomers are added, the sum for diabetic patients (3.54±0.86 ng/ml) is significantly lower than that of healthy subjects (4.13±0.61 ng/ml). However, there is a possibility that the difference just may reflect the difference of average age between the two groups, considering the weak negative correlation between age and serum ATRA level. 

INTERESTINGLY, HbA1c values of T2DM patients correlate positively with serum RAs concentrations (ATRA: r=0.57, p<0.05; 13cRA: r=0.62, p<0.05, Table 3 and Fig. 3a, b). No significant correlation between RAs concentration and other serum lipid indexes (TC and HDLC), except for a correlation between 13cRA and HDLC (r=0.61, p<0.05), was observed. ATRA concentration correlates well with 13cRA concentration (r=0.74, p<0.01). A positive correlation was also observed between HbA1c and TC (r=0.61, p<0.05), while HbA1c did not correlate with age, in spite of the correlation between age and serum ATRA level.

DISCUSSION

In this report, serum ATRA and 13cRA concentrations were quantified by HPLC in healthy volunteers and diabetes mellitus patients. They were estimated at about 2 ng/ml in our system, with comparable reliability to reported values.13) A trace amount of serum 9cRA was detected, but its concentration (about 0.2 ng/ml) was below the quantification limit of our system, with comparable reliability to reported values.13) A trace amount of serum 9cRA was detected, but its concentration (about 0.2 ng/ml) was below the quantification limit of our system. At least the systemic level of 9cRA is not possible biological importance as an endogenous ligand of RXR.

Before the quantification of serum RAs of diabetic patients, we assessed the homeostasis of serum ATRA and 13cRA levels in non-diabetic subjects. Based on the within-day and between-days variances, the concentrations of these two isomers are well maintained, showing little variation. Anomalous increase of serum RAs after a high vitamin A diet was sometimes observed, but the effect was temporary (data not shown and not included in the statistical analysis).

The concentrations of serum ATRA and 13cRA in T2DM patients were rather lower than in normal subjects, but not significantly so. Nevertheless, the sum of the concentrations of the two isomers was significantly lower in diabetic pa-
tients, owing to the good correlation between the two isomers. It is interesting that the lower serum RAs status in T2DM patients contrasts with the report of greater serum retinol level in NIDDM patients,\textsuperscript{6—8)} though the possibility that the lower RAs level just reflects the higher mean of age of the diabetic group cannot be excluded.

The concentrations of serum ATRA of T2DM patients correlated positively with HbA1c values. This contrasts with previous studies on serum retinol level in IDDM patients, in which no correlation between HbA1c and retinol level, despite the significantly lower retinol level, was observed.\textsuperscript{2,3)} Considering the positive correlation of serum RAs levels versus HbA1c together with the negative correlation versus age, there is a possibility that the concentrations of serum RAs may be especially elevated in young and severely (high-HbA1c) diabetic patients. This hypothesis has clinical importance, in view of higher incidence of fetal malformation from high-HbA1c expectant mothers and diabetic mellitus model animals\textsuperscript{14—17)} and the pivotal role of retinoic acid in normal embryonic development.\textsuperscript{18) In addition, higher concentrations of serum RA in such diabetic patients may be an exacerbating factor for diabetic pathogenesis, because it is known that retinoic acid suppresses the differentiation of adipocytes, which may modulate the insulin sensitivity.\textsuperscript{11)} If this hypothesis is correct, the suppression of endogenous retinoic acid actions, for example by retinoid antagonists, may ameliorate diabetic symptoms and complications in some patients. From another viewpoint, a higher concentration of serum RAs may disturb the potency of anti-diabetic agents. Further study using a large number of age-matched subjects will be important for testing these ideas.

Some lipid indexes were also evaluated in this study, but no significant correlation between serum RAs levels and the values in diabetic patients was observed, except for positive correlation between 13cRA and HDLC. Some lipoprotein may be involved in the isomerization of RA to 13cRA or its metabolism.

Even though there are many factors involved in the etiology and pathogenesis of diabetes mellitus and its complications, we propose that uncontrolled concentration of serum RAs in diabetic patients could be of particular importance.

REFERENCES