Urinary Levels of γ -Carboxyglutamic Acid and Its Clinical Significance

Katsuhiro GOTO,^a Seiji KATO,^a Sadaharu SUMIYA,^a Soichiro UEHARA,^b Akio HIRAYAMA,^b Wataru SAKAMOTO,^c Yukiko TOKUMITSU,^d and Masahiko TAKADA^{*,e}

Department of Pharmacy^a and Department of Internal Medicine of Tonan Hospital,^b Kita-1, Nishi-6, Chuo-ku, Sapporo 060, Japan,^b Department of Biochemistry, School of Dentistry^c and Faculty of Pharmaceutical Sciences,^d Hokkaido University, Kita-12, Nishi-6, Kita-ku, Sapporo 060, Japan and Faculty of Pharmaceutical Sciences, Higashi-Nippon Gakuen University,^e Ishikari-Tobetsu, Hokkaido 061-02, Japan. Received April 12, 1993

Urinary γ -carboxyglutamic acid (γ -Gla) levels were determined in healthy subjects of all ages. The urinary γ -Gla levels were highest in infants (0–1 years), then fell in an age-dependent manner, again in subjects reaching a minimum value in adults, then gradually increased over 60 years of age. Urinary γ -Gla levels therefore change markedly with aging. The relationships between the urinary γ -Gla excretion and plasma levels of prothrombin and protein C in patients with various hepatic diseases or diabetes mellitus were examined and compared with those in healthy adults. Both plasma prothrombin and protein C levels were decreased in all patients with liver disease compared with healthy adults. In patients with hepatitis and liver cirrhosis, the decrease did not, however, affect the γ -Gla excretion. In addition, in patients with hepatoma or carcinoma with liver metastases, the urinary γ -Gla levels were increased. In patients with diabetes mellitus, the urinary γ -Gla levels and plasma levels of prothrombin and protein C tended to increase, but this was not significant. The present results indicate that simultaneous measurement of the levels of urinary γ -Gla and plasma prothrombin and protein C is a useful tool for the diagnosis of liver diseases and diabetes mellitus.

Keywords γ -carboxyglutamic acid; prothrombin; protein C; diabetes mellitus; hepatic disease

The residues of γ -carboxyglutamic acid (γ -Gla), which are recently classified as a calcium binding amino acid, have been shown to be present in prothrombin, several clotting factors, anticoagulation factor, protein C and certain proteins such as osteocalcin and atelocalcin involved in bone metabolism.^{1–4} These proteins are synthesized in the liver and are vitamin K-dependent because vitamin K acts as a cofactor in the post-translational carboxylation of specific glutamic acid residues in protein precursors. The vitamin also plays a role in the formation of γ -Gla residues in biologically active proteins.^{5–9}

Free γ -Gla produced by degradation of γ -Gla proteins is well known to be excreted in the urine without any further metabolic degradation.^{10,11} Therefore, the measurement of urinary γ -Gla levels is helpful in characterizing the activity and disorders of the vitamin K-dependent protein metabolizing system.^{12,13} In this study, urinary γ -Gla and plasma levels of prothrombin and protein C in healthy volunteers were determined and compared with those in patients with various hepatic diseases and diabetes mellitus. The clinical significance of the results obtained is discussed.

MATERIALS AND METHODS

Patients and Healthy Volunteers Eighty healthy volunteers and 89 patients participated in this study. Informed consent was obtained from the adult volunteers, all the parents of children investigated and all adult patients. The healthy volunteers were grouped according to age as follows: 0–5 years (12 subjects), 6–15 years (28 subjects), 16–59 years (25 subjects), and 60–75 years (15 subjects), respectively. The 89 patients participating in this study were divided into two groups. Group I consisted of 55 patients with hepatic diseases: 8 patients

with acute hepatitis aged 30–50 years (mean 41 years), 12 patients with chronic hepatitis aged 34–57 years (mean 46 years), 17 patients with liver cirrhosis aged 42–68 years (mean 55 years), 8 patients with hepatoma aged 42–68 years (mean 57 years) and 10 patients with carcinoma with liver metastases aged 54–74 years (mean 65 years). Group II consisted of 34 patients with diabetes mellitus; 7 patients with type I diabetes mellitus aged 37–58 years (mean 44 years) and 27 patients with type II diabetes mellitus aged 52–63 years (mean 48 years). Ten healthy adults aged 36–60 years (mean 48 years) participating in this study represented normal subjects.

Chemicals γ -Gla monoammonium salt was purchased from Calbiochem-Boehringer Co. (La Jolla, Calif. U.S.A.). *o*-Phthalaldehyde (OPA) was obtained from Durrum Chemical Co. (Palo Alto, Calif. U.S.A.) and the OPA reagent was prepared according to the method of Benson and Hare.¹⁴ The creatinine assay kit was obtained from Kainos (Japan). Other materials were all of reagent grade. Water was deionized and then distilled using glass apparatus.

Urine Samples Human urine samples were collected from healthy volunteers immediately after rising in the morning, or collected during a 24 h period from hospitalized patients. The urine was frozen at -20°C , subsequently thawed and centrifuged at $5000 \times g$ for 10 min. The supernatant obtained was diluted with distilled water between 2 to 32 times, and the diluted sample was used for analysis.

HPLC Ten μl aliquots of the samples were injected, and γ -Gla was determined by the method of Kuwada and Katayama.¹⁵ Chromatographic analysis was performed using a Hitachi 655 pump (Tokyo, Japan) for the buffer pump, a Rheodyne Model 720 Injector (Berkeley, Calif. U.S.A.) and an Oriental Motor 3GK25 (Tokyo, Japan) for the OPA reagent pump and a Hitachi 650-10S Fluoro-

Fig. 1. Chromatograms of Amino Acids in Standard Solution and in Urine of Healthy Adults Together with a Calibration Curve of γ -Gla by HPLC

(A) Chromatograms of amino acids. Alanine (Ala), aspartic acid (Asp), glutamic acid (Glu), glycine (Gly), γ -carboxyglutamic acid (γ -Gla). (B) Calibration curve of γ -Gla.

rescence Detector (Tokyo, Japan).

Chromatographic separation was carried out on a 4.6×250 mm column packed with Nucleosil 5SB anion exchange resin ($5 \mu\text{m}$ particles, Macherey-Nagel, Düren, Germany), which was maintained at 47°C in a circulating water bath. The elution buffer was 0.05 M sodium citrate (pH 4.5). The flow rate of the buffer was 0.5 ml/min. The effluent from the column was mixed with OPA reagent and the mixture was carried to a reaction coil in the circulating water bath. The flow rate of the OPA reagent was also 0.5 ml/min. Fluorescence was detected by a Hitachi 650-10S Fluorescence Detector (excitation at 334 nm and emission at 418 nm).

Determination of Urinary Creatinine Urinary creatinine (Cr) is used to standardize urine results.^{12,16} Therefore, the amount of creatinine in the urine samples diluted 20-fold was measured by the enzymatically using a creatinine assay kit and urinary levels of γ -Gla were expressed as nmol/mg Cr.

Determination of Plasma Prothrombin and Protein C Plasma prothrombin was determined by a colorimetric method using S-2238 (Kabi, Vitrum, Sweden) after activation by Echis cartinatus venom. Plasma protein C was determined by an ELISA method (BMY, Germany).

RESULTS

We were able to separate γ -Gla completely from glutamic acid, alanine, aspartic acid, and glycine, although the separation by HPLC was difficult (Fig. 1A). The calibration curve of γ -Gla is linear over a sample range 7.5–60.0 nmol/ml (Fig. 1B). The retention time of both standard and urinary γ -Gla is 16 min. In the γ -Gla determination by HPLC in healthy adults the variability is 5.76% for intra-assay and 13.15% for inter-assay measurements, indicating that the assay displays satisfactory reproducibility. Figure 2 shows the urinary γ -Gla levels in

Fig. 2. Changes in Urinary γ -Gla Levels with Age
Males ($\square$) and females ($\blacksquare$).

healthy subjects of all ages. The levels are 108.6 ± 47.3 nmol/mg Cr in infants (0–5 years), 90.5 ± 25.6 nmol/mg Cr in school children (6–15 years), 62.6 ± 18.7 nmol/mg Cr in adults (16–59 years), and 89.8 ± 21.5 nmol/mg Cr in elderly subjects (over 60 years), respectively. These data indicate that the urinary γ -Gla levels are highest in infants, are attenuated in children, reaching a minimum value in adults and then gradually increase over 60 years of age. Especially, the urinary γ -Gla levels in infants aged 0–1 years are 162.8 ± 37.1 nmol/mg Cr which are much higher than in any other age group. There is no difference between males and females, as far as γ -Gla levels are

TABLE I. Urinary γ -Gla, Plasma Prothrombin and Plasma Protein C Levels in Healthy Adults, Patients with Various Liver Diseases and Patients with Diabetes Mellitus

Diseases	Urinary γ -Gla (nmol/mg Cr)	Plasma prothrombin (μ g/ml)	Plasma protein C (%)
Healthy adults	63.8 $\pm$ 19.2 (n = 10)	77.7 $\pm$ 7.9 (n = 10)	98.9 $\pm$ 5.4 (n = 10)
Liver diseases			
Acute hepatitis	58.7 $\pm$ 15.0 (n = 8)	57.9 $\pm$ 10.2 ^{b)} (n = 8)	74.6 $\pm$ 12.9 ^{b)} (n = 8)
Chronic hepatitis	66.8 $\pm$ 17.0 (n = 8)	56.5 $\pm$ 19.2 ^{b)} (n = 12)	63.9 $\pm$ 17.6 ^{b)} (n = 11)
Liver cirrhosis	50.5 $\pm$ 15.4 (n = 13)	39.2 $\pm$ 17.0 ^{b)} (n = 17)	46.7 $\pm$ 24.7 ^{b)} (n = 17)
Hepatoma	111.4 $\pm$ 40.9 ^{a)} (n = 8)	35.6 $\pm$ 12.4 ^{b)} (n = 7)	33.1 $\pm$ 17.1 ^{b)} (n = 7)
Carcinoma with liver metastases	123.0 $\pm$ 69.9 ^{a)} (n = 10)	56.5 $\pm$ 27.4 ^{a)} (n = 10)	55.6 $\pm$ 29.2 ^{b)} (n = 9)
Diabetes mellitus			
Type I	78.4 $\pm$ 37.7 (n = 4)	89.1 $\pm$ 32.0 (n = 7)	110.2 $\pm$ 19.8 (n = 6)
Type II	79.2 $\pm$ 47.3 (n = 20)	91.0 $\pm$ 21.9 (n = 27)	112.3 $\pm$ 27.8 (n = 27)

Each value represents mean $\pm$ S.D. a) Significantly different from healthy adults at $p < 0.05$. b) Significantly different from healthy adults at $p < 0.01$.

concerned, over all the age groups tested.

To examine the relationship between diseases and γ -Gla protein synthesis, plasma levels of both prothrombin and protein C and, concomitantly, urinary γ -Gla levels were measured in adult patients with a variety of hepatic diseases or diabetes mellitus and also in healthy adults (Table I). In patients with liver cirrhosis, the γ -Gla levels tend to be lower, but the difference is not significant, while the levels of prothrombin and protein C are decreased by 49.5 and 52.8%, respectively, compared with those of healthy adults. In patients with acute and chronic hepatitis, the γ -Gla levels are unchanged, while the levels of prothrombin and protein C are decreased compared with those of healthy adults. In patients with hepatoma and carcinoma with liver metastases, the γ -Gla levels are markedly increased, 74.6 and 92.8%, respectively, while conversely the levels of plasma prothrombin and protein C are decreased compared with those of healthy adults. The levels of urinary γ -Gla, plasma prothrombin, and protein C in patients with type I or type II diabetes mellitus tend to be increased compared with those in healthy adults, but the difference is not significant.

DISCUSSION

The present study demonstrates that urinary γ -Gla levels are highest in the newborn, decrease with age, and then gradually increase after the age of 60. There are a number of papers which have reported the age-dependency of urinary γ -Gla excretion: γ -Gla release into the blood is stimulated during growth because of the increased biosynthesis of osteocalcin in bone.^{10,17)} Both urinary γ -Gla and circulating osteocalcin are increased in growing children.¹⁶⁾ The reduction of skeletal mass with aging results in an increase in urinary γ -Gla excretion.¹⁶⁾ In contrast, there is evidence that the urinary γ -Gla levels in newborn infants are similar to those in adults in spite of the low levels of vitamin K-dependent factors.¹⁸⁾ Gundberg *et al.*¹⁶⁾ reported that the urinary γ -Gla excretion in adults represents normal bone remodeling. Our observations, together with these other reports, suggest that both the increase in the γ -Gla levels in infants and school children and the decrease in adults are concerned with bone turnover. However, more detailed study will be necessary to clarify the relationships between urinary

γ -Gla excretion and bone metabolism.

The plasma levels of both prothrombin and protein C were decreased in all patients with liver diseases tested (cirrhosis, acute or chronic hepatitis, hepatoma, and carcinoma with metastases), although there was difference in size of the decrease in liver diseases. The γ -Gla excretion in hepatitis and cirrhosis was unchanged, while the γ -Gla excretion in hepatoma and carcinoma with liver metastases was increased. It has been reported that in patients with hepatitis or cirrhosis, normal prothrombin was decreased and abnormal prothrombin, which is undetectable in normal persons, was increased, while in patients with hepatocellular carcinoma, normal levels of prothrombin are maintained.¹⁹⁾ In contrast, patients with acute hepatitis or cirrhosis had low, but detectable, abnormal prothrombin, suggesting that a defect in vitamin K-dependent carboxylation occurs in hepatic tumors and results in the synthesis of abnormal prothrombin.^{20,21)} We do not know if the urinary γ -Gla excretion in different patients originates from a particular kind of vitamin K-dependent protein since we did not determine the abnormal prothrombin level.

Mannucci and Vigano²²⁾ reported that in 60 healthy adults, there were no differences in plasma protein C levels in terms of age or sex. In addition, they demonstrated that in patients with chronic liver diseases (chronic hepatitis and liver cirrhosis), protein C levels were lower than in healthy subjects. These results are consistent with our observations.

It has been reported that in children with type I diabetes mellitus, serum osteocalcin levels were significantly decreased because of decreased bone formation.²³⁾ Diabetes mellitus is associated with a hypercoagulable state involving abnormal increase in raising of several vitamin K-dependent clotting factors.²⁴⁾ These observations are consistent with our own observations that plasma levels of prothrombin and protein C tend to be increased in patients with diabetes mellitus and this is accompanied by an increase in urinary γ -Gla excretion. The levels of vitamin K in biological materials are not routinely determined at the moment. In the present study, we have shown that the simultaneous assay of urinary γ -Gla levels together with prothrombin, abnormal prothrombin, and protein C provides a good index of abnormal metabolic states of vitamin K-dependent

proteins and that this is a useful tool to aid the profound diagnosis of liver diseases and diabetes mellitus.

REFERENCES

- 1) J. Stenflo, P. Fernlund, W. Egan, P. Roestorff, *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 2730 (1974).
- 2) J. B. Lian, M. Skinner, M. J. Glimcher, P. M. Gallop, *Biochem. Biophys. Res. Commun.*, **73**, 349 (1976).
- 3) J. B. Lian, E. L. Prien, M. J. Glimcher, P. M. Gallop, *J. Clin. Invest.*, **59**, 1151 (1977).
- 4) R. G. Discipio, E. W. Davie, *Biochemistry*, **18**, 899 (1979).
- 5) J. Stenflo, *J. Biol. Chem.*, **251**, 355 (1976).
- 6) P. W. Hauschka, P. A. Friedman, H. P. Traverss, P. M. Gallop, *Biochem. Biophys. Res. Commun.*, **71**, 1207 (1976).
- 7) J. B. Lian, P. A. Friedman, *J. Biol. Chem.*, **253**, 6623 (1978).
- 8) P. A. Friedman, P. V. Hauschka, M. A. Shia, J. K. Allace, *Biochim. Biophys. Acta*, **583**, 261 (1979).
- 9) R. J. Levy, J. A. Zenker, J. B. Lian, *J. Clin. Invest.*, **65**, 563 (1980).
- 10) M. Shiraki, I. Akiguchi, H. Itoh, T. Kaneko, M. Matsuo, *Nippon Ronengakkai Zasshi*, **20**, 128 (1983).
- 11) D. V. Shah, J. K. Tews, A. E. Harper, J. W. Suttle, *Biochim. Biophys. Acta*, **539**, 209 (1978).
- 12) R. J. Levy, J. B. Lian, *Clin. Pharmacol. Ther.*, **25**, 562 (1979).
- 13) S. Uehara, K. Honjo, A. Hirayama, K. Goto, *Chemotherapy*, **35**, 254 (1989).
- 14) J. R. Benson, P. E. Hare, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 619 (1975).
- 15) M. Kuwada, K. Katayama, *Anal. Biochem.*, **177**, 259 (1981).
- 16) C. M. Gundberg, J. B. Lian, P. M. Gallop, *Clin. Chim. Acta*, **128**, 1 (1983).
- 17) P. D. Delmas, D. Stenner, H. W. Warner, K. G. Mann, B. L. Riggs, *J. Clin. Invest.*, **71**, 1316 (1983).
- 18) L. Sann, M. Leclercq, M. Fouillit, M. C. Chapius, A. Bruyere, *Clin. Chim. Acta*, **142**, 31 (1984).
- 19) R. A. Blanchard, B. C. Furie, M. Jorgensen, S. F. Kruger, B. Furie, *N. Engl. J. Med.*, **305**, 242 (1981).
- 20) H. A. Liebman, B. C. Furie, M. J. Tong, R. A. Blanchard, K. J. Lo, S. D. Lee, M. S. Coleman, B. Furie, *N. Engl. J. Med.*, **310**, 1427 (1984).
- 21) D. V. Shah, J. A. Engelke, J. W. Suttie, *Blood*, **69**, 850 (1987).
- 22) P. M. Mannucci, S. Vigano, *Lancet*, **II**, 462 (1982).
- 23) K. Kruse, U. Kracht, *Eur. J. Pediatr.*, **145**, 27 (1986).
- 24) A. Hughes, B. A. McVerry, L. Wilkinson, A. H. Goldstone, D. Lewis, A. Bloom, *Acta Haematol.*, **69**, 254 (1983).