Enzyme immunoassay of urinary mevalonic acid and its clinical application

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We have developed an enzyme immunoassay for mevalonic acid (MVA), using a specific monoclonal antibody. The intra- and interassay coefficients of variation calculated on two urine samples were 3.3% and 3.4%, respectively, in the intraassay precision test and 3.5% and 6.9% in the interassay evaluation. Pravastatin, a 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor, was administered to nine healthy men, and in all cases, their MVA excretion rates then decreased. The more MVA that was excreted in the urine before the pravastatin administration, the greater a reduction of MVA excretion was observed. The daily MVA excretions in healthy men (n = 120) and women (n = 105) were 2.32 μmol/day (SD, 0.82 μmol/day) and 1.85 μmol/day (SD, 0.47 μmol/day), respectively. In streptozotocin-induced diabetic rats (n = 14), the plasma cholesterol concentrations and MVA excretion rates were increased, and a positive correlation was observed between the plasma cholesterol and the urinary MVA concentrations.

Mevalonic acid (MVA; for structure, see Fig. 1A) is the immediate product of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme of cholesterol biosynthesis. The MVA concentrations in plasma have been reported to be highly correlated with the whole body cholesterol synthesis determined by the sterol balance method, suggesting that MVA is a good indicator of the in vivo rate of cholesterol biosynthesis (1). MVA has a low molecular weight and is hydrophilic; it is excreted in the urine, and the urinary MVA is excreted in proportion to its concentration in plasma (1, 2). It is thus thought that in vivo cholesterol biosynthesis can be evaluated by the measurement of MVA in the urine. Gas chromatography–mass spectrometry (3–7) and radioenzyme methods (1, 8) have been used for the measurement of MVA; however, the former method is complicated and requires expensive equipment, and the latter is also complex and has problems associated with radioisotopes. We therefore obtained a specific monoclonal antibody against MVA and developed an enzyme immunoassay using this antibody and peroxidase-labeled antigen (9).

In the present study, we measured the MVA in human and rat urine with the new enzyme immunoassay to evaluate the clinical importance of the concentrations of urinary MVA. It has been observed that the amount of MVA excretion was reduced by the administration of an HMG-CoA reductase inhibitor (6, 10, 11). To test this finding, we also examined the changes in the MVA excretion after the administration of pravastatin, one of the HMG-CoA reductase inhibitors, to healthy men. In studies using diabetic dogs (12, 13), cholesterol biosynthesis and urinary MVA were increased. Studies of a diabetic rat model revealed that the cholesterologenesis in the gut was increased along with increases of plasma cholesterol and triglyceride concentrations (14–18); however, the urinary MVA in a diabetic model has not yet been reported. We therefore measured the urinary MVA concentrations of streptozotocin (STZ)-induced diabetic rats.

Materials and Methods

EXTRACTION OF URINARY MVA

Urinary MVA was extracted by our previously described methods (9). A SEP-PAK PS-1 column (Waters) was prewashed with 4 mL of methanol and 0.5 mol/L NaCl (pH 1.0). For the lactonization of MVA, 2 mL of human urine or 0.5 mL of rat urine was mixed at a 10:1 ratio with methanol:water (90:10).
6 mol/L HCl, left at room temperature for 40 min, and then applied to the column. After the column was washed with 3.6 mL of a saturated ammonium sulfate solution (pH 1.5), the MVA was eluted with 8 mL of 0.1 mol/L HCl containing 15 mL/L methanol. The eluate was mixed with 8 mL of the saturated ammonium sulfate solution (pH 1.5) and applied to another SEP-PAK PS-1 column. After the column was washed with 1 mL of water (pH 2.9), the MVA was eluted with 8 mL of water (pH 2.9) containing 25 mL/L methanol. Then, 2 mol/L NaOH (305 μL) was added to the eluate. After 40 min at room temperature, 2 mol/L phosphate (213 μL) was added to make a 50 mmol/L phosphate buffer (pH 7.4) for 6 h at room temperature and used as the enzyme-labeled antigen (Fig. 1D) was conjugated to horseradish peroxidase (POX). A succinimidyl ester of the MVA derivative synthesized in our laboratory (Fig. 1B) was reacted with keyhole limpet hemocyanin in 50 mmol/L phosphate buffer (pH 7.4) for 6 h at room temperature and used as the immunogen (Fig. 1C). Likewise, another succinimidyl ester of the MVA derivative (Fig. 1D) was conjugated to horseradish peroxidase (Toyobo) and used as the enzyme-labeled antigen (Fig. 1E). BALB/c mice were immunized with the MVA derivative-keyhole limpet hemocyanin conjugate, and an anti-MVA antibody-producing hybridoma (MHM-9H) was obtained. The antibody from the hybridoma was purified by Protein A-Sepharose CL-4B (Pharmacia Fine Chemicals) chromatography. The antibody was identified as IgG-κ by the Mono Ab Screen Id/Sp kit (Zymed Laboratories) and confirmed to be monoclonal by a linear Scatchard plot of the binding data with 14C-MVA.

**PREPARATION OF ANTI-MVA ANTIBODY AND ENZYME-LABELED ANTIGEN**

The immunogen and enzyme-labeled antigen were prepared by the N-succinimidyl ester method (19). A succinimidyl ester of the MVA derivative synthesized in our laboratory (Fig. 1B) was reacted with keyhole limpet hemocyanin in 50 mmol/L phosphate buffer (pH 7.4) for 6 h at room temperature and used as the immunogen (Fig. 1C). Likewise, another succinimidyl ester of the MVA derivative (Fig. 1D) was conjugated to horseradish peroxidase (Toyobo) and used as the enzyme-labeled antigen (Fig. 1E). BALB/c mice were immunized with the MVA derivative-keyhole limpet hemocyanin conjugate, and an anti-MVA antibody-producing hybridoma (MHM-9H) was obtained. The antibody from the hybridoma was purified by Protein A-Sepharose CL-4B (Pharmacia Fine Chemicals) chromatography. The antibody was identified as IgG-κ by the Mono Ab Screen Id/Sp kit (Zymed Laboratories) and confirmed to be monoclonal by a linear Scatchard plot of the binding data with 14C-MVA.

**ENZYME IMMUNOASSAY**

A solution (100 μL) of authentic MVA or a sample to be tested was added to the wells of a 96-well plate (Nunc) that was coated with goat anti-mouse IgG. Then, 20 ng of anti-MVA antibody in 50 mmol/L phosphate buffer (pH 7.2) containing 1 g/L bovine serum albumin (buffer A, 50 μL) and 1.25 ng of peroxidase-labeled antigen (Fig. 1B) in buffer A (50 μL) were added. The plate was incubated at 4°C for 16 h. After the plate was washed two times with 300 μL of buffer A, 250 μL of substrate solution containing 0.4 mmol/L 3,3′,5,5′-tetramethylbenzidine and 1.4 mmol/L H2O2 (pH 4.5) was added, and the enzyme reaction was performed at room temperature for 15 min. The reaction was stopped by the addition of 0.5 mol/L H2SO4, and the absorbance at 450 nm was measured.

**ADMINISTRATION OF PRAVASTATIN TO HEALTHY MEN**

The subjects were nine healthy men (25–50 years old) who work in our laboratory, and informed consent was obtained from each. All urine samples were collected at proper intervals during a period lasting from 1245 on day 1 to 1830 on day 3. At 1830 on day 2 and at 0930 on day 3, 10 mg of pravastatin (oral; Sankyo Co.) was administered to each subject after supper and breakfast.

**MEASUREMENT OF URINARY MVA IN HEALTHY SUBJECTS**

The subjects were 120 male and 105 female office workers who underwent a health checkup by the protocol described elsewhere (20). They had no history of diabetes and took no drugs that influenced lipid metabolism, such as a hypolipidemic drug. They provided 24-h urine samples.

**DIABETIC RAT MODEL EXPERIMENT**

Male Sprague–Dawley rats (8 weeks old; n = 14) were injected intravenously with 50 mg/kg streptozotocin (Sigma Chemical Co.) in 0.1 mol/L citrate–0.2 mol/L phosphate buffer (pH 4.5). Urine was collected during a 20.5-h period (from 1200 of day 1 to 0930 of day 2) in a metabolic cage. Heparinized blood was collected from the jugular vein. Food containing <5 mg/kg cholesterol was given to the rats during the study. The blood glucose concentrations were measured by a Tide glucose analyzer (Bayer Sankyo). The plasma cholesterol was measured by a Daiya-color TC kit (Toyobo). Control rats of the same strain and age (n = 10) were maintained under the same conditions.

**STATISTICS**

All values are mean ± SD except those in Figs. 3 and 5, which are mean ± SE. The significance of differences between paired means or between unpaired means was calculated by the Student t-test. Paired means were used in the experiment with pravastatin, and unpaired means were used in the other experiments. The linear regression analysis was performed by the least-squares method. P < 0.05 was considered significant.
Results

CROSS-REACTIVITY OF MONOCLONAL ANTIBODY
As shown in Fig. 2, the detection range of the enzyme immunoassay was 1.5–170 pmol/test (IC_{50} = 15 pmol/test). The cross-reactivity of the antibody with MVA analogs contained in the biological fluid, such as glutaric acid, 3-methyl-glutaric acid, and 3-hydroxy-3-methyl-glutaric acid was <0.001%. The antibody cross-reacted at <0.001% with pravastatin, which has an MVA-like structure.

MEASUREMENT SYSTEM OF URINARY MVA
The overall recovery of radioactive MVA added to 2 mL of human urine was 89.9% ± 1.0% (n = 24) after the column extraction procedures. When a known amount of authentic MVA (0.74–2.57 μmol/L) was added to human urine and subjected to the assay procedure, the added MVA was recovered with an average yield of 101.7% ± 7.1% (n = 10). The intra- and interassay coefficients of variation calculated on two urine samples were 3.3% and 3.4% (mean = 47.5, 22.0 pmol/test, n = 20) in the intraassay precision evaluation and 3.5% and 6.9% (mean = 46.4, 21.6 pmol/test, n = 10) in the interassay evaluation.

MEASUREMENT OF URINARY MVA EXCRETION IN HEALTHY SUBJECTS
Pravastatin was administered to nine healthy men, and their urine was collected and subjected to the enzyme immunoassay. The MVA excretion was reduced after the first administration (i.e., at 1830 on day 2) and the second administration (0930 on day 3) in all subjects (Fig. 3). Before the administration (from 1245 on day 1 to 1845 on day 2), the MVA excretion peak was observed between morning and noon in two subjects; however, in others the diurnal changes of MVA excretion were not clear. In all subjects, the daily urinary MVA excretion after pravastatin administration was reduced. The daily MVA excretion was reduced by 36.6% (P < 0.001) on average (Table 1). The preadministration value of the daily MVA excretion was positively correlated with the reduced MVA value after administration (P < 0.05) (Fig. 4). Table 1 shows the values of daily MVA excretion in the healthy male and female populations. The MVA excretion in the men was significantly (P < 0.001) higher than that in the women.

URYINARY MVA OF DIABETIC RAT MODEL
The blood glucose concentrations of the rats were increased from day 1 after STZ injection and reached ~5 g/L (500 mg/dL) at day 3 after injection. In the control rats, the blood glucose concentration was ~1500 mg/L (150 mg/dL) throughout the experiment. Although it was not a significant difference, from day 2 after the injection, the amounts of MVA excretion in the diabetic rats (0.40 ± 0.11 μmol/day, n = 14) tended to be higher than those of the control rats (0.33 ± 0.11 μmol/day, n = 10; Fig. 5A).

| Table 1. Urinary MVA excretion in healthy subjects. |
|---------------------------------|-----------------|------------------|
| Age, mean ± SD (range)         | MVA, μmol/day, mean ± SD (range) |
| Men (n = 9)                    | 31.0 ± 7.9 (25–50) | 2.32 ± 0.65 (1.50–3.58) |
| (Before pravastatin administration) |                    |                  |
| Women (n = 10)                 | 37.9 ± 11.0 (19–58) | 1.85 ± 0.47 (0.73–3.19) |

| Table 2. Urinary MVA excretion in diabetic rats. |
|---------------------------------|-----------------|------------------|
| Age, mean ± SD (range)         | MVA, μmol/day, mean ± SD (range) |
| Men (n = 15)                   | 37.3 ± 10.8 (21–58) | 2.32 ± 0.82 (0.70–4.76) |
| (Before pravastatin administration) |                    |                  |
| Women (n = 15)                 | 37.9 ± 11.0 (19–58) | 1.85 ± 0.47 (0.73–3.19) |

Fig. 2. Calibration curve for MVA.
The standard enzyme immunoassay of MVA was performed in the presence of various amounts of unlabeled MVA (●). Various amounts of glutaric acid (X), 3-methyl-glutaric acid (▲), 3-hydroxy-3-methyl-glutaric acid (▲), and pravastatin (†) were also subjected to the standard enzyme immunoassay.
On day 6 after the injection, significantly \((P < 0.001)\) higher MVA excretion rates were observed in the diabetic rats \((0.61 \pm 0.14 \mu mol/day)\) compared with the control rats \((0.38 \pm 0.08 \mu mol/day)\). In the diabetic rats, the plasma total cholesterol concentrations were significantly \((P < 0.05)\) increased at 2 weeks after the injection \([986 \pm 180 \text{ mg/L} (98.6 \pm 18.0 \text{ mg/dL})]\) compared with the concentrations before STZ \([851 \pm 77 \text{ mg/L} (85.1 \pm 7.7 \text{ mg/dL})]\); the total cholesterol concentrations were not significantly changed in the control rats (Fig. 5B).

At 2 weeks after the injection day, the plasma cholesterol concentrations were positively correlated with the urinary MVA concentrations \((r = 0.535, P < 0.01; \text{Fig. 6A})\), and still correlated after 1 month \((r = 0.728, P < 0.001; \text{Fig. 6B})\).

### Discussion

Urinary MVA is considered to be a marker of cholesterol biosynthesis in vivo. We therefore established an enzyme immunoassay of urinary MVA, using a specific antibody \((9)\). The values of human urinary MVA obtained by this immunoassay correlated well with those obtained by the conventional radioenzyme method \((r = 0.969; \text{Fig. 7})\) \((9)\).

The average amounts of daily MVA excretion measured by our method were \(2.32 \pm 0.82 \mu mol/day\) and \(1.85 \pm 0.47 \mu mol/day\) in healthy men and women, respectively. Similar values of MVA in human urine have been obtained by the radioenzyme method \((10, 21)\) and by gas chromatography–mass spectrometry \((4, 6, 7)\). Our method of urinary MVA extraction is simple and does not require an internal standard such as radiolabeled MVA because the reproducibility of urinary MVA recovery by this method was found to be excellent \((89.9\% \pm 1.0\%)\). High precision and low intra- and interassay coefficients of variation of the urinary MVA values were also obtained by this method.

To evaluate the clinical importance of MVA measurement, pravastatin was administered to nine healthy men, and the changes in their urinary MVA excretions were observed. Although pravastatin has an MVA-like structure, we believe that the urinary MVA measurement was not influenced by pravastatin in urine because the cross-reaction with the anti-MVA antibody was <0.001%, and most pravastatin is excreted with feces \((22)\). As shown in Fig. 4, the MVA excretion was significantly reduced after the pravastatin administration. The higher the MVA excretion rate was before the pravastatin administration, the more the excretion of MVA after the pravastatin administration was reduced. This result suggested that an HMG-CoA reductase inhibitor may more efficiently reduce the cholesterol synthesis in a patient who has a high urinary MVA concentration because urinary MVA is considered to reflect cholesterol synthesis in vivo. Previous reports indicated that above-average HMG-CoA reductase inhibitor responders had substantially higher
mean pretreatment plasma concentrations of MVA than below-average responders (23). In addition, HMG-CoA reductase inhibitor is thought to be more effective when cholesterol synthesis in vivo is high. Our results suggest that the measurement of urinary MVA would be clinically useful as a marker of HMG-CoA reductase indication for reductase inhibitor is thought to be more effective when below-average responders (23).

We also investigated the significance of urinary MVA in an STZ-induced diabetic rat model. In the diabetic rats, the urinary MVA concentrations were twice of those of the control rats at 2 weeks after the STZ injection. Together with the increased concentrations of urinary MVA, the plasma cholesterol concentrations of the diabetic rats increased, and a positive correlation was observed between the urinary MVA and plasma cholesterol concentrations. In a diabetic dog model, the MVA excretion was increased together with both HMG-CoA reductase activity and cholesterologenesis in the intestine and liver (12–13). Both HMG-CoA reductase activity and cholesterologenesis have reported to be substantially increased in vivo. Therefore, the increase of urinary MVA in the diabetic rats reflects the accelerated cholesterologenesis in the gut. These results indicate that urinary MVA is a good marker of HMG-CoA reductase activity and cholesterol biosynthesis in vivo. Because significant changes in the MVA concentration were observed in the rat model, the measurement of urinary MVA is expected to be useful for research regarding lipid metabolism in animal models.

The present MVA assay method is very simple compared with the conventional gas chromatography–mass spectrometry and radioenzyme methods. We may further simplify the extraction procedure and measure a large number of samples automatically. The assay will also be useful for the diagnosis and monitoring of disorders affecting lipid metabolism, such as diabetes, hyperlipidemia, and atherosclerosis.

References