Urinary Excretion Levels of Hydroxylysin Glycosides in Osteoporotic Patients

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The urinary excretion levels of hydroxylysine glycosides [Hyl-Gs; O-β-d-Galactopyranosylhydroxylysine (GH) and 2-0-α-d-glucopyranosyl-O-β-d-galactopyranosylhydroxylysine (GGH)], provide a new index of collagen metabolism. The determination of urinary hydroxylysine glycoside excretion levels was applied to 20 osteoporotic patients and 208 healthy control subjects (69 females and 139 males) in order to evaluate the conditions of bone collagen metabolism. Urinary Hyl-Gs were analyzed by the method of pre-column fluorescent derivatized HPLC. Compared to the age and sex-adjusted control, the urinary excretion levels of hydroxylysine glycosides, especially GH, in osteoporotic patients were significantly higher than in the age and sex-adjusted control, and the urinary GGH/GH ratio was lower. Sixteen of the 20 patients whose Hyl-Gs levels we followed exhibited significantly lower values after 200 d compared to the initial levels. These results suggest that clinical therapeutics affected the bone resorption and, therefore, demonstrated the usefulness of determining urinary excretion levels of Hyl-Gs for evaluating the conditions of bone metabolism.

Keywords hydroxylysine glycoside; urinary excretion level; osteoporosis; bone resorption; collagen metabolism; biomedical marker

Osteoporosis and the associated increased incidence of fractures pose serious health problems for the elderly, especially women. Biological markers, such as serum alkaline phosphatase and urinary hydroxyproline excretion, are routinely used to assess the rate of bone formation and resorption, respectively. But both tests are strongly influenced by non-osseous metabolism and are subject to interference from various systemic disorders. Furthermore, the release of hydroxyproline at the stage of collagen biosynthesis, including procollagen degradation, is also complicated. Consequently, using urinary hydroxyproline excretion levels for the indirect evaluation of bone resorption does not provide adequate information on the rate of bone collagen breakdown. Therefore, the development of some new method for assessing skeletal mass conditions is desired for predicting fracture risk based on osteoporosis.

Recently, the urinary excretion levels of hydroxylysine glycosides (Hyl-Gs) have been proven to be a reliable new index of collagen metabolism. O-β-d-Galactopyranosylhydroxylysine (GH) and 2-0-α-d-glucopyranosyl-O-β-d-galactopyranosylhydroxylysine (GGH) are the glycosides, and are located in the exact position of an α-chain which contains mainly type I and II collagen in bone and skin. The GGH/GH content ratio of the collagen is varied according to the type of collagen; for example, the most common content ratio is about 2 in skin collagen and less than 0.5 in bone collagen. Moreover, these glycosides are released with collagen degradation and excreted in urine, not to be metabolized in the liver or kidney. The urinary Hyl-Gs excretion levels are not affected by diet, and the GH excretion level is inversely correlated with bone mineral density. Therefore, by observing the urinary excretion levels of Hyl-Gs and the urinary GGH/GH ratio, it can be made clear which type of tissue is being degraded.

In the present paper, in order to evaluate the conditions of bone collagen metabolism, determination of the urinary excretion levels of Hyl-Gs were applied to osteoporotic patients and the excretion levels were compared to those of age and sex-adjusted control groups.

MATERIALS AND METHODS

Urine Samples The first micturition urine after arising was collected from 20 ambulatory patients (19 females and 1 male ranging in the age from 66—83 years old) with osteoporosis. On the basis of their back pain and/or radiological investigation, all patients were diagnosed as having osteoporosis. These 20 patients were in good health except for osteoporosis, and had no recognizable disease or history of the use of drugs known to produce osteoporosis. They had already been administered, for 1 or 2 months, an oral preparation of vitamin D, intra muscular administration of calcitonin to improve their back pain, and rehabilitative exercise therapy, if necessary. In order to estimate the condition of bone resorption associated with the effectiveness of those clinical treatments, urine samples were collected from 16 of these 20 patients about every two weeks for 200 d. Two of the 16 patients (1 female and 1 male) were followed since the time of their first medical examination, and were medically untreated with respect to osteoporosis before their hospitalization.

As controls, urine samples were obtained from 208 healthy persons (69 females and 139 males) ranging from 40 to 89 years of age who had no back pain or lumbago. Their urine was also collected at their first micturition. The utility of the first micturition urine after arising instead of 24-h urine has already been described. After
collection, urine samples were stored at $-20^\circ C$ and kept frozen until the time of analysis.

**Determination of Urinary Hyl-Gs** Hyl-Gs were determined by HPLC following the procedure published previously. Briefly, 100 $\mu l$ of urine sample or Hyl-Gs standard solution was added to the mixture of 100 $\mu l$ of carbonate buffer (0.3 M, pH 9.5) and 200 $\mu l$ of 40 mm dansyl chloride (DNS-CI) in acetonitrile in a screw-capped Pyrex tube with a Teflon liner. The mixture was kept at 60 $^\circ C$ for 20 min, and the reactant was loaded into an HPLC apparatus equipped with a gradient system. Urinary Hyl-Gs excretion levels were expressed as $\mu$mol/g creatinine and the value of each patient was the means of the two values of two successive days. Urinary creatinine was measured by the colorimetric procedure based on the Jaffe method using a clinical test kit (Creatinine-test Wako; Wako Pure Chemical Industries, Ltd. Japan).

**Statistical Methods** All results were expressed as the mean ± standard deviation (S.D.). Comparisons for significance were made using a paired or unpaired Student’s $t$-test, and all $p$ values were < 0.05.

**RESULTS AND DISCUSSION**

**Urinary Excretion Levels of Hyl-Gs in Osteoporotic Patients Compared to Healthy Controls** First of all, it was necessary to obtain a large number of values from healthy persons to determine the control levels of urinary Hyl-Gs excretion prior to the determination of urinary excretion levels of Hyl-Gs in osteoporotic patients. As shown in Fig. 1, the urinary excretion levels of Hyl-Gs in the female control groups increased with age. On the other hand, the male control groups exhibited a relatively constant level above age 50. The GH excretion levels tended to higher in females in their sixties, seventies and eighties than those in males, but the difference was not statistically significant, suggesting that bone resorption was accelerated by aging. Moreover, the high excretion levels of urinary GH in females over 60 are explained by the higher bone resorption at an older age partly due to menopause. The means and standard deviations of urinary Hyl-Gs excretion levels and urinary GGH/GH ratios for different age groups of females and males are given in Table I. The GGH/GH ratio shows no age dependent difference in the control. The normal values obtained for different age groups are useful for the situational evaluation of abnormal collagen metabolism, especially in the bone and skin.

In contrast, the urinary excretion levels of Hyl-Gs in the osteoporotic patient group (Table I) exhibited a high and very wide range of values compared to that of the age and sex-adjusted controls. It was suggested that these results were partly based upon their different osteoporotic states. More remarkable are the urinary excretion levels of GH in osteoporotic patients, which are significantly higher than those of the control. These results were also

**Table I. Urinary Excretion Levels of Hyl-Gs in Different Age Groups of Healthy Control and Osteoporotic Patients**

<table>
<thead>
<tr>
<th>Age (year)</th>
<th>GGH ($\mu$mol/g cre.)</th>
<th>GH ($\mu$mol/g cre.)</th>
<th>GGH/GH ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Patient</td>
<td>Control</td>
</tr>
<tr>
<td>Females</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40–49</td>
<td>18.07 ± 6.0 (15)</td>
<td>17.76 ± 9.4</td>
<td>1.09 ± 0.22</td>
</tr>
<tr>
<td>50–59</td>
<td>24.38 ± 9.9 (13)</td>
<td>21.40 ± 7.5</td>
<td>1.46 ± 0.31</td>
</tr>
<tr>
<td>60–69</td>
<td>32.06 ± 9.1 (18)</td>
<td>25.73 ± 7.5</td>
<td>1.27 ± 0.33</td>
</tr>
<tr>
<td>70–79</td>
<td>32.61 ± 10.2 (12)</td>
<td>26.13 ± 7.2</td>
<td>1.27 ± 0.35</td>
</tr>
<tr>
<td>80–89</td>
<td>36.21 ± 10.6 (5)</td>
<td>28.57 ± 10.3</td>
<td>1.37 ± 0.44</td>
</tr>
<tr>
<td>Males</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40–49</td>
<td>25.01 ± 3.4 (5)</td>
<td>16.37 ± 3.0</td>
<td>1.55 ± 0.25</td>
</tr>
<tr>
<td>50–59</td>
<td>26.10 ± 7.5 (6)</td>
<td>17.46 ± 2.3</td>
<td>1.47 ± 0.64</td>
</tr>
<tr>
<td>60–69</td>
<td>32.88 ± 11.9 (46)</td>
<td>21.51 ± 8.7</td>
<td>1.61 ± 0.43</td>
</tr>
<tr>
<td>70–79</td>
<td>31.78 ± 9.6 (72)</td>
<td>21.08 ± 7.7</td>
<td>1.57 ± 0.36</td>
</tr>
<tr>
<td>80–89</td>
<td>32.25 ± 14.4 (105)</td>
<td>21.86 ± 8.8</td>
<td>1.50 ± 0.37</td>
</tr>
</tbody>
</table>

$a$ Values are means ± S.D.  $b$ Number of samples determined.  $c$ The difference compared to the control (unpaired $t$ test, $p<0.05$).
Fig. 2. Changes in Urinary Excretion Levels of Hyl-Gs in Osteoporotic Women
Data of 16 ambulatory osteoporotic women after 200 d compared to the initial levels. The left panel (a) depicts GH levels and right panel (b) GGH levels. All the patients were administered a preparation of vitamin D orally and/or calcitonin intra muscularly during the test period.

Fig. 3. Changes in Urinary Hyl-Gs Levels Over Time during the Clinical Treatment
Two ambulatory patients were investigated; (a) female, 79 years old, (b) male, 83 years old. (○) and (●) denote GGH and GH, respectively. 0 d indicates the day when the patient took the first medical examination. Over time, the patients were administered with a preparation of vitamin D orally, and calcitonin intra muscularly if necessary. The horizontal dashed lines represent the mean ± 1 S.D. of the urinary GH excretion levels of age-adjusted controls.

supported by the fact that the GGH/GH ratio in osteoporotic patients decreased compared to the control. Thus, it has been proven that urinary Hyl-Gs levels, especially GH, increased in osteoporotic patients.

Figure 2 shows the urinary excretion of Hyl-Gs in 16 patients at the start and after 200 d. The urinary excretion levels of GGH and GH gradually decreased over the time, and were significantly lower after 200 d compared to the initial levels ($p < 0.05$). Throughout the testing period all the patients were administered with a preparation of vitamin D orally and/or calcitonin intra muscularly, GH, in particular, apparently decreased due to the effects of drug therapeutics and rehabilitative exercise; it should be noted that some of the 16 patients had their drug administration broken off by their physician after 200 d because of the improvement in their condition or the cessation of complaints.

We followed two patients (1 female and 1 male) from the time of their first medical examination for osteoporosis. The patients were medically untreated with respect to osteoporosis before their hospitalization. Figure 3 shows the time course of their urinary Hyl-Gs excretion levels during the ambulatory medical treatment and consultation. Urinary GGH and GH levels gradually decreased, and especially, urinary GH levels returned to the mean ± 1 S.D. of the age and sex-corrected control levels within 150 d. These results suggest that clinical therapeutics decrease the bone resorption, lowering the urinary Hyl-Gs, especially GH, excretion levels.

To date, it has been demonstrated that urinary GGH excretion levels increase in patients with skin disease, such as burns or erythema multiforme, and GH excretion levels increase in patients with bone disease such as osteomalacia or Paget’s disease. On the other hand, urinary excretion levels of Hyl-Gs follow the same physiological variations as urinary hydroxyproline; the level is not influenced by dietary protein intake, and GH excretion levels are inversely correlated with bone mineral density. To our knowledge, with this background, this is the first comprehensive study of urinary Hyl-Gs excretion in patients with osteoporosis compared to age and sex-adjusted control groups, and provides a means of evaluating bone metabolism from the viewpoint of the conditions of bone collagen metabolism.

Because present therapeutic efficiency is not very powerful or effective, osteoporosis has to be prevented or medically treated prior to its distinct onset. To date, such methods as single and dual photon absorptiometry, dual energy X-ray absorptiometry and quantitative computed tomography (QCT), provide only the information on the bone mineral content in the osteoporotic state. Consequently, a more sensitive and specific biochemical test or biomedical marker based on the bone collagen metabolism will be needed.

In this paper, we have shown the utility of charting the urinary excretion levels of Hyl-Gs as one of a new index of bone mass conditions; urinary excretion levels of GH increase in osteoporosis and reflect the effects of clinical
treatment on bone turnover, suggesting that they could be of clinical value in the screening of women at risk for osteoporosis.

Acknowledgments The authors thank Mr. Motohiko Kato, Mr. Masanori Miura and Mr. Kennichiro Konno for their technical assistance.

REFERENCES AND NOTES

1) A part of this work was presented at the 113th Annual Meeting of the Pharmaceutical Society of Japan, Osaka, March 1993.