High Predictivity of Galactosyl-hydroxylysine in Urine as an Indicator of Bone Metastases from Breast Cancer

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We measured the urinary excretion of galactosyl-hydroxylysine (GH) and hydroxyproline in two groups of women with breast cancer, with (M+, n = 24) and without (M0, n = 30) clinical, scintigraphic, or radiological evidence of bone metastases. Both these compounds are excreted in larger amounts in the M+ group than in the M0 patients. However, GH, which is a specific marker for bone collagen, provides better predictivity for bone metastases than does hydroxyproline: 92% sensitivity and 90% specificity vs 74% and 79%, respectively, for hydroxyproline.

Additional Keyphrases: hydroxyproline · amino acids · amino acid glycosides · collagen metabolism

The measurement of hydroxyproline in urine is commonly used to evaluate collagen metabolism (1, 2). High concentrations of this amino acid have been observed in urine in many pathological conditions affecting skin and bone (3–5).

It is generally agreed that increases of hydroxyproline excretion reflect an abnormally high rate of collagen turnover. There are, however, serious limitations to the use of this marker because (a) only about 10–25% of the hydroxyproline of degraded collagen is excreted in urine (6), and (b) its measurement does not provide information on the specific tissue where the collagen breakdown takes place, e.g., bone vs soft tissues (7).

Collagen contains two glycosides of hydroxylysine: glucosyl-galactosyl-hydroxylysine (GGH) and galactosyl-hydroxylysine (GH) (7). Because GH is sevenfold more abundant than GGH in bone collagen, GH is considered bone-tissue specific (8). Both glycosides are almost completely (50–100%) excreted in urine during collagen degradation (7), and their excretion, which is not influenced by diet, is increased in physiological and pathological conditions characterized by high collagen turnover (9).

Relatively few clinical conditions involving collagen breakdown have been studied by using these hydroxylysine glycosides as biochemical markers, probably because of the difficulty of the determinations involved. Our group recently developed a simple and reliable method to measure urinary GH by HPLC (10). We found an inverse correlation between GH and bone mineral density, as measured by quantitative computed tomography, and used the HPLC results to identify osteoporotic women (11, 12). We undertook the present study to determine the efficiency of GH, in comparison with hydroxyproline, for predicting the risk of bone metastases in patients with breast cancer.

Materials and Methods

Patients

Two age-matched groups of women with breast cancer were selected for this study: 24 patients with (M+) clinical, radiological, and scintigraphic evidence of bone osteolytic or osteoblastic metastases and 30 without (M0) such evidence. The patients had no previous history of any hepatic or skeletal disease and were not receiving drugs that might influence skeletal metabolism, e.g., corticosteroids, calcitonin, or diuretics. Clinical laboratory data and descriptions of the patients will be published elsewhere (manuscript in preparation).

Reagents

The following reagent solutions were prepared: standard didansylated GH (prepared as previously reported (13)), 30 μmol/L; sodium carbonate, 300 mmol/L; dansyl chloride solution in dimethyl ketone, 10 g/L; buffer A: per liter, 50 mmol of sodium acetate (pH 6.3), 125 mL of acetonitrile, and 50 mL of propan-2-ol; buffer B: 50 mmol of sodium acetate (pH 6.5), 500 mL of acetonitrile, and 10 mL of propan-2-ol. Didansylated GH (30 μmol/L) was used to evaluate the amount of GH excreted in urine (external standard).

Apparatus

We used a Model 344 HPLC (Beckman Instruments, Fullerton, CA) connected to a Spectra/Glo fluorometer (Gilson Medical Electronics, Inc., Middleton, WI). For measurement, the excitation wavelength was 366 nm, the emission wavelength 490 nm. The area of the peaks was calculated by an HP 3390 automatic integrator (Hewlett-Packard, Avondale, PA). Reversed-phase HPLC was performed with a 4.6 cm × 7 cm UltraSphere 3-μm (particle size) octadecylsilyl column. The two solvent systems used for the stepwise gradient (Table 1), buffer A and buffer B, are described above. The flow rate was maintained at 1 mL/min.

Procedures

Urine collection. A 24-h urine sample was collected from each patient for measurement of creatinine, hydroxyproline, and GH. The collection started after three days of a collagen-free diet. Borate (1 g/L) was added to the urines to

Table 1. Gradient Elution Program

<table>
<thead>
<tr>
<th>Gradient start time, min</th>
<th>Buffer B, %</th>
<th>Gradient duration, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10</td>
<td>—</td>
</tr>
<tr>
<td>0.1</td>
<td>45</td>
<td>—</td>
</tr>
<tr>
<td>18.0</td>
<td>100</td>
<td>3.5</td>
</tr>
<tr>
<td>27.0</td>
<td>10</td>
<td>0.1</td>
</tr>
</tbody>
</table>

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prevent bacterial growth. The samples were stored at -70 °C until analyzed.

Sample preparation. About 4 mL of each 24-h urine was centrifuged at 200 × g for 10 min to remove sedimentable material. The supernate was then derivatized with dansyl chloride according to the method of Gray (14). In brief, we mixed 100 μL of urine with 100 μL of Na2CO3 (0.3 mol/L) and added this to 200 μL of dansyl chloride (10 g/L) in dimethyl ketone. After incubation at 60 °C for 30 min and filtration through an HV 0.45-μm (pore size) filter (Millipore, Yonezawa, Japan), 20 μL of the mixture was injected into the HPLC system.

Between- and within-run precision. To determine between-run precision, we dansylated 16 samples of the same urine on 16 different days, then analyzed by HPLC. Within-run precision was determined by dansylating a sample of urine and subjecting it to seven separate chromatographic separations. We adopted the same procedure to calculate the between- and within-run precision of hydroxyproline determinations.

Creatinine and hydroxyproline determinations. Creatinine was measured according to the Jaffé method with a kit from Biochemia (Boehringer Mannheim GmbH, Mannheim, F.R.G.); hydroxyproline was quantified according to the procedure of Kivirikko et al. (15).

Statistics. Statistical differences between the groups were evaluated by Student's t-test.

Results

Preliminary to this study, we calculated total (between-run) and within-run precision of the assays (Table 2). Within-run CVs for GH and hydroxyproline were 1.7% and 11.0%, respectively; total CVs were 7.0% and 3.0%. The mean values of GH and hydroxyproline, excreted by the two groups of patients, were as follows. The patients in the M<sub>0</sub> group excreted 12.5 (SD 2.9) μmol of GH per gram of creatinine, the same as that already reported in a previous study for a group of normal subjects of the same age (11). The excretion rate increased in the M<sub,+</sub> group to 30.6 (SD 4.5) μmol/g of creatinine, a statistically significant difference (P < 0.01). The values for hydroxyproline excretion were also significantly (P < 0.01) higher in the M<sub>+</sub> group (165.5 μmol/g of creatinine, SD 79.1) than in the M<sub>0</sub> group (93.3 μmol/g of creatinine, SD 39.1). No correlation between the two markers was found.

One way to derive the discriminatory value (threshold) for GH and hydroxyproline tests is to plot the specificity vs the sensitivity. From Figure 1 it appears that the best threshold value is 16.0 μmol/g of creatinine for the GH test and 120 μmol/g of creatinine for hydroxyproline. By adapting those threshold values, the sensitivity and specificity obtained are as reported in Table 3, which shows also the predictive values of positive and negative results and the value of efficiency.

Discussion

The precision data (Table 2) show that the analytical methods to measure urinary GH and hydroxyproline provide CVs on the same order of magnitude: this makes possible the comparison between the two markers and allows one to draw correct conclusions from the information obtained with these tests.

Both GH and hydroxyproline were increased in the urine of the patients of M<sub>+</sub> group, the mean values being about 1.7 times greater than those in the urine of the M<sub>0</sub> patients. However, the SD for hydroxyproline determinations is much higher than for GH. The explanation for this may be the large variability in the degradation of collagen from soft tissues (6).

The lack of correlation between the two markers within the groups may be explained in the same way: the influence of diet can be excluded because the patients were on a collagen-free diet before the urine collections.

The previous finding was that GH was a good marker to identify patients affected by a high turnover of bone collagen is thus confirmed. In fact, post-menopausal women at high risk of osteoporosis were as well identified by GH as by quantitative computed tomography (11). In collaboration with Baylink's group (16), we performed a study to detect patients affected by Paget's disease: eight biochemical markers were compared for their discriminatory power, and GH received the highest z-score.

Hydroxyproline was also used as a marker, but its z-score was less good, even though the values correlated with those of GH test. On the contrary, for post-menopausal women (12) and in the present investigation, there was no correlation between the two biochemical markers.

Consequently, the two markers apparently correlate

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Table 2. Precision of Assays for GH and Hydroxyproline

<table>
<thead>
<tr>
<th></th>
<th>GH</th>
<th>Hydroxyproline</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Between-run precision</strong> (n = 16 each)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean, μmol/g creat.</td>
<td>9.4</td>
<td>120</td>
</tr>
<tr>
<td>SD, μmol/g creat.</td>
<td>0.86</td>
<td>9.6</td>
</tr>
<tr>
<td>CV, %</td>
<td>7.0</td>
<td>8.0</td>
</tr>
<tr>
<td><strong>Within-run precision</strong> (n = 7 each)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean, μmol/g creat.</td>
<td>9.6</td>
<td>122</td>
</tr>
<tr>
<td>SD, μmol/g creat.</td>
<td>0.74</td>
<td>13.42</td>
</tr>
<tr>
<td>CV, %</td>
<td>7.7</td>
<td>11.0</td>
</tr>
</tbody>
</table>

Fig. 1. Receiver operating characteristic (ROC) curve for GH (●) and hydroxyproline (Hyp. Δ · Δ)

Threshold values shown are μmol/g of creatinine

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only when very high rates of bone collagen breakdown characterize the disorder (as in Paget's disease). In fact, if bone is the tissue affected in the disorder, the contribution of hydroxyproline from soft tissues is dominated by the hydroxyproline released from bone.

Considering the main point of this study, i.e., the ability of GH as a marker to discriminate between metastases and a nonmetastatic condition, we refer to Figure 1. The best threshold value is that which is closest to the intersection of the axes. As shown by Figure 1, and clearly stated in Table 3, which summarizes all the data, GH is able to recognize up to 92% of the cases with tumor dissemination in bone; when the tumor is not metastasized, the discriminatory power is 90%. Interestingly, three patients in the M₀ group had a GH/creatinine ratio >16 μmol/g; one month later, they became positive for bone metastases, as determined with roentgenography (data not shown). We conclude that GH provides better information than hydroxyproline in monitoring patients at risk for bone metastases.

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References


Table 3. Predictive Value (%) of GH and Hydroxyproline for Bone Metastases

<table>
<thead>
<tr>
<th>Threshold values, μmol/g creatinine</th>
<th>Predictive value of</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Pos. result</th>
<th>Neg. result</th>
<th>Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactosyl-hydroxylysine</td>
<td>16.0</td>
<td>92</td>
<td>90</td>
<td>88</td>
<td>93</td>
<td>91</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>120.0</td>
<td>74</td>
<td>79</td>
<td>74</td>
<td>79</td>
<td>77</td>
</tr>
</tbody>
</table>

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