Bone Tissue Resorption Markers in Metastatic Involvement of the Skeleton

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Clinical significance of intermolecular relationships of bone matrix collagen pyridinoline and deoxypyridinoline in the urine is assessed in 137 cancer patients with metastases to the bones and 16 without metastatic involvement of the bone. Bone tissue resorption markers were studied by solid-phase extraction and high performance liquid chromatography and excretion was expressed as the ratio to urine creatinine. The levels of collagen pyridine bonds were significantly higher in cancer patients with metastases to bones than in patients without metastases and in control group consisting of 137 normal subjects. In addition, urine levels of pyridinoline and deoxypyridinoline in cancer patients without metastases to bones were significantly higher than in controls. A significant increase in urinary excretion of pyridine bonds was observed in bone involvement in patients with malignant tumors of different localization: breast, lungs, and prostate. The data indicate a possibility of using collagen pyridine bonds for early detection of metastatic destruction of the skeleton.

Key Words: *collagen pyridine bonds; pyridinoline; deoxypyridinoline; bone tissue, metastases*

Various markers of bone tissue resorption are used for the diagnosis and monitoring of osteoporosis, Paget's disease, hyperthyrosis, and other metabolic diseases involving destruction of the skeleton. Pyridinoline (PD) and deoxypyridinoline (DPD), or collagen pyridine bonds (CPB) excreted in the urine with collagen fragments are widely used in clinical practice [4,5,12,14].

Study of the structure of collagen whose destruction is typical of bone tissue resorption showed that the stability of collagen matrix is provided by irreversible intermolecular bonds forming between some, predominantly essential, amino acids in collagen polypeptide chain. Analytical studies showed that a common feature of these bonds is the pyridine ring appearing during formation of collagen fibrils. The identified intermolecular collagen bonds are hydroxylysilPD and lysylPD forming on the basis of lysine and amino acid residues, unique for collagen molecules [12,13,15]. In accordance with their structure, collagen cross bonds were denoted PD and DPD.

Further studies demonstrated that CPB are present only in extracellular collagen fibrils and are characteristic of differentiated matrix of solid connective tissue: bone, cartilage, and dentine, but absent from skin or soft tissue collagen, which favorably differs them from hydroxyproline, whose specificity as a bone resorption marker is relatively low because of its high prevalence in tissues of many types. In addition, up to 40% of urinary hydroxyproline is formed due to degradation of newly formed collagen and its precursors, this requiring strict collagen-free diets for measuring hydroxyproline. An important advantage of CPB is that both PD and DPD are almost completely excreted with urine in free (40%) or peptide bound (60%) form upon degradation of collagen matrix, while hydroxyproline can be reutilized in collagen synthesis [4,12,15].

Study of CPB distribution in different connective tissues showed that osseous tissue is the main source of PD in biological liquids of the organism. This type

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of bonds is represented in cartilaginous tissue and tendons, but active metabolism of bone tissue in comparison with other connective tissues indicates that PD is excreted with urine due to normal or abnormal destructive processes in the bones. Its analog DPD is found solely in collagen of bone tissue, where the PD:DPD ratio is 4:1. This ratio is stable in the urine of adults. DPD is responsible for 20-22% of total level of excretion of CPB [4,12,14,15]. This ratio shifts toward increase in the relative content of PD only in articular diseases of different origin, whose differential diagnosis is based on assessment of the PD/DPD ratio because of absolute predominance of type I CPB in cartilaginous tissue [15].

Recent studies of molecular mechanisms of metastases and development of new approaches to the treatment of metastases in the bone prompts the search for sensitive and specific markers allowing timely diagnosis and monitoring of metastases into the bones. Publications about CPB as criteria of bone tissue resorption in metastatic involvement of the skeleton are scanty and the studies are as a rule performed in small groups of patients [3,7,9,10]. Our purpose was to assess the significance of measuring collagen fragments containing CPB in the urine of cancer patients for the diagnosis of metastases of malignant tumors of different localization to the bones.

MATERIALS AND METHODS

A total of 137 cancer patients with metastases to the bones were observed, 120 of these with breast cancer (aged 29-75 years), 6 with prostatic cancer (48-70 years), 4 with lung cancer (68-70 years), and 7 with unknown primary focus (46-62 years). Metastases to the bones were diagnosed by scanning of the skeleton with ^{99m}Tc technophore with polymethylene diphosphonate and subsequent x-ray examination. Sixteen patients with tumors of the same localizations without metastases in the bones were a reference group. Control group consisted of 137 normal subjects (124 women aged 28-75 years, and 13 men aged 47-74 years), matching for age and sex to cancer patients with metastases in the bones.

CPB were measured in the second spontaneous morning urine portion after its hydrolysis by an equivalent volume of 12 M hydrochloric acid at 107°C for 18 h in hermetically sealed tubes in order to isolate CPB from collagen fragments excreted with urine. PD and DPD levels in the urine were measured by high performance liquid chromatography [11] using Gilson Aspec (Anachem) automated system and Microsorb C18 chromatographic column (10 cm×4.6 mm, Rainin Instr.). PD and DPD detection is based on natural fluorescence of their component pyridine. It was measured by a fluorescence monitor Shimadzu RF-530. Preliminary solid-phase extraction was carried out on columns packed with CC-31 microgranulated cellulose placed between two polyethylene filters with 20-μm pores. After centrifugation at 13,500*g* for 2 min, urine hydrolysates (0.5 ml) were applied onto columns which were then washed with 8 ml mixture of mobile phase (primary butyl alcohol:acetic acid:water in 4:1:1 ratio) at a flow rate of 0.75 ml/min. For removing the butyl phase, the columns were washed with tetrahydrofurane (0.5 ml), after which CPB were eluted by means of paraionic solvent of heptafluorobutyric acid (0.44 ml). Mixing of solvents and applied specimens in an Aspec automated system was prevented by using a 50-μl air segments between different liquids. Separation of PD, DPD, and internal reference placed at the beginning of each series of specimens from other fluorescent components was realized by high performance liquid chromatography with 2 solvents: A) 0.01 M aqueous solution of heptafluorobutyric acid and B) 0.01 M heptafluorobutyric acid in 0.75% water solution of acetonitryl (flow velocity 1 ml/min). Extracted specimens of urine and reference specimens were automatically applied onto chomatographic column in solvents A and B in 83:17 ratio. A two-step gradient of acetonitryl was attained by proportional increase of the proportion of solvent B from 17 to 20% for 7 min and then to 25% during subsequent 5 min, followed by a sharp increase of its concentration to 70%. Complete cycle (28.5 min) was finished by preparing the column to separation of the next specimen during gradual decrease in the concentration of solvent B to the initial concentration of 17%. In parallel with this, the fluorescence of eluates was measured under the following spectroscopy conditions: stimulation at 295 nm and emission at 400 nm. Quantitative analysis of isolated CPB fractions excreted with urine was automated, with due consideration for the size of PD and DPD peaks of the internal reference which was added into the system for the detector calibration; their concentrations corresponded to 120.3 and 39.8 nmol/liter. Results were expressed in units nmol/mmole urinary creatinine; for this, creatinine concentrations were measured in each native urine specimen. In addition, calcium concentrations were measured in patients' sera. Creatinine and calcium concentrations were measured by optimal spectrophotometric methods in a Hitachi-911 automated analyzer.

RESULTS

Study of urinary excretion of collagen fragments containing CPB in cancer patients with metastases

Table 1. PD and DPD Levels in Urine of Cancer Patients with and without Metastases into Bones ($X \pm m$)

Parameters, nmol/mmol creatinine	Control group (n=137)	Cancer patients	
		with metastases to bones (n=137)	without metastases to bones (n=16)
PD	36.3±0.8 (17.6—60.4)	172.7±10.2 (42.2—853.4)	70.1±5.3 (44.0—114.1)
DPD	10.3±0.31 (4.9—26.2)	47.2±3.4 (7.8—378.8)	17.7±1.8 (9.4—30.4)

Note. All values significant (p<0.001) in comparison with the control and with patients without metastases. Here and in Tables 2 and 3: variation range is indicated in parentheses.

into the bones showed a significant increase in the concentrations of PD and DPD in comparison with age- and sex-matched healthy controls (p<0.001) and cancer patients without metastases into the bones (p<0.001, Table 1). The mean increase in urinary excretion of PD and DPD in patients with metastases in comparison with the control was almost the same (4.8 and 4.6 times, respectively). In comparison with cancer patients without verified metastases, this increase was lower: 2.5 times for PD and 2.7 times for DPD. Table 1 demonstrates variability in these parameters in cancer patients with metastases. In the control group of 137 normal subjects, PD concentrations were 17.6-60.4 and DPD 4.9-26.2 nmol/mmol creatinine, which is in line with the data of foreign scientists [15,17]; in cancer patients with metastases these parameters varied in a wider range: PD 42.2-853.4 nmol/mmol and DPD 7.8-378.8 nmol/mmol. The PD concentration was increased in comparison with the normal threshold value (60.4 nmol/mmol) in 132 (96.3%) out of 137 patients. DPD was higher than normally (26.2 nmol/mmol) in 100 (73%) out of 137 patients. An increase (2-14 times) of PD excretion was detected in 81 (61.4%) out of 132 patients, of DPD in 40 (40%) out of 100 patients. In other cases an increase in CPB excretion in comparison with the control was much lower (less than twofold).

Analysis of data in cancer patients without metastases showed a significant increase in the concentrations of collagen fragments in comparison with normal controls (Table 1). The mean increase of PD and DPD levels in comparison with normal values was smaller (1.9 and 1.7 times) than in patients with bone metastases. Increased PD and DPD levels in the urine of cancer patients without pathological changes in the skeleton in comparison with normal excretion of these substances were found in 8 out of 16 (50%) and 3 out of 16 (18.7%), respectively, the increase being negligible in comparison with the upper normal threshold value. In patients with metastases to the bones, CPB concentration was rather high (PD 853.4 and DPD 378.8 nmol/mmol) — at least 14 times higher than the maximum normal value, while in patients without metastases to the bones, the maximal values of PD and DPD (114.1 and 30.4 nmol/mmol) were only 1.9 and 1.2 times higher than in normal controls, respectively.

Analysis of urine from patients with tumors of different localizations with metastases into the bones showed no essential differences in the mean concentrations of CPB in patients with breast, lung, and prostatic cancer (Table 2). The levels of PD and DPD were the highest in patients with breast cancer metastases to the bones, which can be explained by the greatest number of such patients in comparison with other patients and by the type of metastases. Bone tissue resorption is the predominant component of osteolytic metastases, characteristic of breast cancer, while in osteoblastic metastases of prostatic cancer,

Table 2. PD and DPD Concentrations (nmole/mmole Creatinine) in Urine of Patients with Malignant Tumors of Different Localizations with and Without Metastases into Bones ($X \pm m$)

Tumor localization	Control		With metastases		Without metastases	
	PD	DPD	PD	DPD	PD	DPD
Mammary gland	36.1±0.9 (17.6—60.4)	10.3±0.3 (4.9—26.2)	173.6±11.3 (42.2—853.4)	47.3±3.8 (7.8—378.8)	65.1±6.1 (45.8—102.5)	16.1±2.2 (9.4—30.4)
Lung	34.9±6.4 (25.6—48.7)	9.9±1.4 (6.2—16.4)	171.9±32.6 (100—266.9)	41.2±4.1 (31.4—54.8)	88.7±15.1 (61.7—114.1)	23.3±3.2 (18.8—29.4)
Prostate	38.9±3.0 (26.6—58.7)	10.4±1.0 (7.1—16.4)	140.1±26.5 (51—220.5)	42.8±9.6 (12.1—71)	70.5±19.0 (44—91.5)	19.5±5.3 (9.6—27.9)

bone tissue formation prevails. Similarity of destructive processes in the bones in osteoporosis and in malignant tumor involvement suggested a high specificity of CPB as markers of osteolytic metastases. It is impossible to differentiate between the lytic and sclerotic metastases in the bones on the basis of collagen cross bonds [2,19].

Increased urinary excretion of PD and DPD in patients with bone metastases of different pathogenesis can be explained by mixed pattern of pathological changes in the skeleton in malignant tumors of different localization, specifically, in breast and prostatic cancer. Recent comparative x-ray and pathomorphological studies demonstrate manifestations of osteolysis and osteosclerosis in metastases of solid malignant tumors, which may result from the effects of a variety of paracrine factors produced by malignant tumors on bone tissue [2,6,18]. CPB as components of bone tissue collagen matrix are specific markers of resorption, characteristic of primarily osteolytic metastases, and therefore analysis of findings in breast cancer patients was of special interest.

Comparative analysis of urinary CPB in 120 patients with breast cancer with verified metastases and in 10 patients without bone metastases showed a significant increase in PD excretion ($p<0.001$) and DPD ($p<0.001$) in metastatic involvement of the bones (2.7 and 2.9 times, respectively). The mean increase of excretion in comparison with 120 age-matched healthy controls was even greater: 4.9 and 4.6 times, respectively. Despite a slight difference in the levels of PD and DPD, the mean levels of their excretion in patients with breast cancer without metastases to the bones significantly ($p<0.01$) surpassed the corresponding values in the control group. It is noteworthy that results of comparative study of bone tissue resorption markers in cancer patients with metastases and without pathologic changes in the skeleton are contradictory. Some authors note the absence of significant differences in urinary excretion of CPB in cancer patients without metastases to the bones and in normal subjects [1,6,20]. Others report a tendency toward an increase in the urinary concentrations of PD and DPD in cancer patients without bone involvement [7,8,19]. One of the possible mechanisms is the indirect effect of primary malignant tumors on the production of paracrine factors stimulating bone tissue resorption [2,6,18]. Despite the absence of clinical signs of metastases in the bones, the involvement of the skeleton cannot be ruled out in such patients because of well known difficulties in early diagnosis of metastases.

Patients with breast cancer metastases to the bones were characterized by great variability of CPB values in comparison with patients without bone metastases and controls. We analyzed the relationship between urinary CPB levels and blood serum calcium levels in patients with metastases. Despite the direct effects of various paracrine factors on calcium metabolism in cancer patients, serum calcium concentration is one of the most significant biochemical signs of activity of bone destruction in osteolytic metastases. Hypercalcemia (Ca level over 2.6 mmol/liter in the blood serum) was detected in 24 (20%) out of 120 patients with breast cancer. CPB excretion was increased in comparison with normal values for PD (60 nmol/mmol) and DPD (26.7 nmol/mmol) in virtually all patients with hypercalcemia. The only exclusion was a patient with DPD of 18.6 nmol/mmol. On the other hand, in the patients with normal serum calcium levels (Ca no higher than 2.6 mmol/liter), PD excretion was increased in 92 (95.8%) out of 96 and DPD in 64 (66.7%) out of 96 patients. Distribution of patients by serum calcium concentrations (Table 3) showed a significant increase of PD and DPD levels in patients with hypercalcemia (Ca level at least 2.6 mmol/liter) in comparison with patients with normal calcium levels in the serum (Ca no higher than 2.6 mmol/liter). Moreover, the highest levels of CPB were found in patients with hypercalcemia (Table 3).

Table 3. CPB Excretion and Serum Calcium Concentrations in Patients with Breast Cancer Metastases to the Bones ($X \pm m$)

Parameters	Normocalcemia (n=96)	Hypercalcemia (n=24)
Calcium, mmol/liter	2.4±0.01 (2.15—2.6)	3.0±0.08 (2.62—3.96)***
PD, nmol/mmol	154.8±10.3 (42.2—564.1)	248.7±35.0 (69.8—853.4)**
DPD, nmol/mmol	40.3±2.6 (7.8—129.5)	75.2±15.1 (18.6—378.8)*

Note. *$p<0.05$, **$p<0.02$, ***$p<0.001$ in comparison with normocalcemia.

The detected regularities consisting in notably increased urinary excretion of collagen fragments containing CPB in patients with metastases of tumors of different localizations to the bones are in line with many publications [1,8,16,19,20]. Many scientists proved that PD and, even more so, DPD are specific markers of bone tissue resorption. It is probable that the detected increase of CPB concentration in the urine of cancer patients indicates a manifest lytic process not only in patients with breast or lung cancer metastases to the bones, but also indicates involvement of the skeleton in patients with prostatic cancer. According to our findings, PD and DPD are sensitive markers of metastatic destruction of osseous

tissue, because increased CPB excretion is observed in patients with both extensive and local involvement of the bones.

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