Quantitation of Biochemical Markers of Bone Resorption Following Strontium-89-Chloride Therapy for Metastatic Prostatic Carcinoma

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The urinary production of pyridinium collagen cross-links, pyridinoline (PYD) and deoxypyridinoline (DPD), has been correlated to increased bone resorption in patients with neoplasms. This study investigated the production of these compounds in patients with metastatic prostate carcinoma who received palliative treatment that did and did not include ^{89}Sr-chloride therapy. **Methods:** Urinary production of PYD and DPD was measured by high-performance liquid chromatography and natural fluorescence detection methods. The urine from several age-matched groups of patients was examined for these compounds including healthy controls (n = 20), patients with early-stage (Stage A-B) prostate carcinoma (n = 8), patients with metastatic prostate carcinoma treated with conventional analgesic and radiotherapeutic palliation (n = 20), patients with metastatic disease who underwent ^{89}Sr-chloride therapy (n = 20) and patients with mild Paget's disease (n = 5). Patients were also monitored for urinary PYD and DPD production for a 6-mo interval after a palliative intervention. **Results:** Elevated PYD and DPD ($p < 0.05$) concentrations were measured in patients with metastatic and nonmetastatic prostate cancer and Paget's disease. The urinary production of these compounds remained unchanged for 6 mo after ^{89}Sr-chloride therapy for symptomatic osseous metastases. However, the patients who did not undergo ^{89}Sr-chloride therapy exhibited a two-fold increase in PYD and a four-fold increase in DPD above controls during that interval. **Conclusion:** PYD and DPD are sensitive and specific bone resorption markers which demonstrate a slowing of bone resorption after palliative ^{89}Sr-chloride therapy in patients with bone metastases.

Key Words: strontium-89-chloride therapy; osseous metastases; radionuclide therapy; bone resorption; pyridinium cross-link

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The management of recurrent and progressive pain in patients with osseous metastases represents a substantial clinical challenge. Skeletal metastases occur in up to 85% of patients with advanced prostate, breast and lung carcinoma as well as other malignancies (*1,2*). Approximately 70% of patients with metastatic prostatic carcinoma respond auspiciously to hormonal manipulation that utilizes orchiectomy, estrogens, antiandrogens and luteinizing hormone-releasing hormone agonists. Unfortunately, many patients who initially respond favorably to hormonal treatment inevitably relapse (*3*). Additionally, hormonal manipulation or cytotoxic chemotherapy are of very limited benefit to patients who relapse (*4,5*). The palliative treatment of patients with osseous metastases who fail hormonal manipulation, or are not candidates for such treatment, is local or wide-field (hemi-body) radiotherapy to the symptomatic site(s). Systemic ^{89}Sr-chloride therapy has also demonstrated the capability of palliating pain from osseometastases secondary to prostatic carcinoma (*6–10*).

Palliation of symptoms and improvement in the quality of life represent the major therapeutic goals in the treatment of metastatic bone disease with ^{89}Sr-chloride. The efficacy of this treatment is generally monitored by measuring indirect and net health outcomes that are related to metastatic disease in general (*7,10*). For instance, diminished radionuclide uptake in a metastatic lesion or failure of disease progression on bone scintigraphy represent beneficial intermediate health outcomes. Similarly, improved patient mobility and decreased narcotic or non-narcotic analgesic requirements represent beneficial net health outcomes. Although these outcomes are subjectively useful in monitoring disease progress, they represent a partial evaluation of disease state because they may not correlate with objective disease measures (falling prostate specific antigen (PSA), resolving bone scan, etc.). This discordance is problematic because it prevents an association between objective measures and patient symptoms for the purpose of designing effective palliative strategies. In this regard, an objective evaluation of treatment efficacy would assist in optimizing treatment planning and offering choices to patients with varying symptoms and disease stages.

A reliable and specific marker of bone resorption could potentially serve as an objective index of the extent of metastatic disease and the effect of a particular therapy on the disease course. Urinary calcium (*11*) and hydroxyproline (*12*) concentrations have been used as markers of bone resorption. Their routine use for the evaluation of treatment response and disease progression is limited by several important factors. Specifically, urinary calcium excretion reflects the difference between the rate of bone resorption and formation, and in patients with primarily sclerotic metastatic disease it may be within the normal range. This would potentially result in a false-negative study in patients with breast cancer or other malignancies which produce predominantly sclerotic bone disease. Similarly, hydroxyproline levels are potentially confounded by the contribution of collagen synthesis, complement activation, malignant destruction of soft tissues and diet. Consequently, false-positive hydroxyproline levels might be detected in a patient with stable disease.

Several potential markers of bone turnover have recently been reported. One class of molecules is correlated to bone formation and includes procollagen type I C-terminal and type III N-terminal peptides. Precursors of type I collagen measured in the serum of growth hormone-deficient children correlate with bone formation rates (*13*). In a recent investigation, serum procollagen I and III peptide levels were slightly elevated or normal in patients who responded to ^{89}Sr-chloride therapy for bone metastases whereas those who obtained no benefit from ^{89}Sr-chloride demonstrated highly abnormal levels (*14*). These

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TABLE 1
Patient Characteristics

Variable	EBRT Group* n	89-Sr Group* n
Sites of metastasis		
Hips	4	3
Hips + spine	10	11
Hips + spine + ribs	3	4
Spine + ribs	3	2
Serum PSA [μg/liter]	466 ± 133	490 ± 101
Prostate tumor gleason score		
4–7	13	13
8–10	4	3
Prostate volume (cm3)	40 ± 17	32 ± 19
Time from diagnosis (yr)	3.2 ± 1.1	3.8 ± 1.4
Time from symptom onset to palliation therapy (wk)	2.3 ± 1.7	3.0 ± 1.9
Karnovsky performance score	79 ± 8	85 ± 5

*n = 20 patients; all values reported as mean ± s.d. EBRT = external beam radiation therapy.

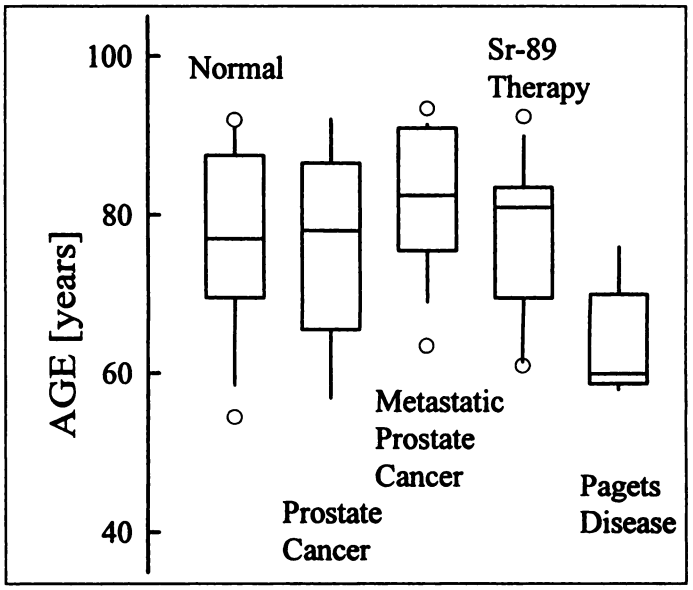

FIGURE 1. Age range of the patients in each of the groups. The mean ± s.d. of ages (yr) were: normal (76.6 ± 12.2), early prostate carcinoma (76.1 ± 13.3), metastatic prostate carcinoma (81.7 ± 9.5), metastatic carcinoma treated with ^{89}Sr-chloride therapy (77.4 ± 9.9) and Paget's disease of bone (64.2 ± 7.6). There was no statistically significant difference in the mean ages of patients in different groups. The 5th and 95th %ile values are indicated by open circles where these values were outside the range.

peptide levels did not correlate with PSA or CA15-3 antigen levels which themselves did not correlate with the clinical response.

Another class of potential bone turnover markers are associated with bone resorption and include pyridinium cross-links of collagen (*15*) and peptides derived from the nonhelical (telopeptide) regions of type I collagen (*16*). The extracellular matrix of bone and collagen is substantially stabilized by covalent cross-links between adjacent collagen fibrils. These cross-links provide tensile strength to bone and are released from mature collagen during bone resorption and excreted in the urine in free (40%–50%) and peptide-bound (50%–60%) forms. Two important nonreducible cross-links, which are amino acid derivatives, have been isolated from bone: pyridinoline (PYD), which is a 3-hydroxypyridinium derivative that is largely present in extracellular collagen fibrils of bone and cartilage (*17*) and deoxypyridinoline (DPD), which is another derivative found almost exclusively in bone (*18*). These cross-links have been proposed as specific markers of bone turnover because they are not present in the collagen of normal skin, fascia and ligaments. Histomorphometric and radioisotopic studies have demonstrated a high correlation between urinary concentrations of PYD and DPD and the rate of bone resorption (*19,20*)

Increased concentrations of PYD and DPD have been reported in the urine of breast and prostate cancer patients (*21*). PYD and DPD have also been investigated for monitoring metastatic bone disease (*22,23*) and the effect of pamidronate in patients with metastatic disease (*24*). The present investigation was undertaken to examine the urinary excretion of PYD and DPD during palliative therapy using ^{89}Sr-chloride. Moreover, the principal aims of this study were to confirm that these derivatives were abnormal in patients with metastatic prostatic carcinoma and to determine what levels were achieved in the course of ^{89}Sr-chloride therapy to predict clinical outcome and to optimize clinical management.

MATERIALS AND METHODS

Patients

Patient characteristics are described in Figure 1 and Table 1. The latter includes demographic data regarding patients who randomly underwent conventional palliative external beam irradiation for skeletal metastases or ^{89}Sr-chloride therapy. Forty patients with at least one skeletal metastatic lesion (^{99m}Tc-MDP bone scintigraphy) secondary to histologically proven prostatic carcinoma (Stage D) were examined in accordance with university research policy. All of the patients had been treated by orchiectomy and/or pharmacological hormonal therapy but had progressive pain from one or more metastatic sites. Patients who had previously received local irradiation for palliation of skeletal metastases different from the progressive symptomatic lesions were also included in the analysis. None of the patients had a second malignancy. All had adequate initial hematological function including a mean platelet count of 190 ± 70 (× 10^9/liter) and leukocyte count of 4.8 ± 1.5 (× 10^9/liter). Half of the patients received a single systemic treatment of 50–80 μCi/kg body weight ^{89}Sr-chloride (average dose of 4.3 mCi). These patients underwent subsequent follow-up visits at 1, 3 and 6 mo after ^{89}Sr-chloride therapy. Routine clinical chemistry was performed at those visits. Twenty-four hour urine samples were also collected for subsequent biochemical analysis at the time of follow-up visits.

The other half (n = 20) of this study population underwent external beam radiation therapy (EBRT) (35 Gy in 14 fractions or 30 Gy in 10 fractions) for palliation of a painful bony metastasis. Healthy patients with no history of carcinoma (n = 20) were included as negative controls and patients with biopsy-proven prostatic adenocarcinoma (Stage A-B) but without skeletal metastases (^{99m}Tc-MDP bone scintigraphy) were also investigated (n = 8). Five patients (n = 5) with Paget's disease of bone diagnosed by radiographic evidence and ^{99m}Tc-MDP bone scintigraphy were also studied and served as positive controls of PYD and DPD urinary excretion. The mean ages of these groups of patients were comparable (Fig. 1) and not statistically significantly different.

Biochemical Analysis

The urine collected at follow-up was acidified with the addition of 1.5 ml of normal hydrochloric acid (3% HCl) for every 20 ml of urine and stored at −20°C for subsequent measurement of PYD, DPD, calcium and creatinine. Urine samples were hydrolyzed overnight and filtered by CF1 cellulose columns (recovery > 90%) before high performance liquid chromatography (HPLC) separa-

tion. The method for the separation of PYD and DPD has been described in detail elsewhere (*15,24*). Briefly, the assay used ion-paired reverse phase HPLC protocol. A 5 μm octadecylsilane (**ODS**) column (100 × 4.6 mm) was used to separate PYD and DPD. The column temperature was maintained at 12°C with a water jacket. The column was eluted with a mobile phase that consisted of 25 m*M* sodium formate, 5 m*M* 1-octanesulfonic acid and 1 m*M* ethylenediaminetetraacetic acid adjusted to pH 3.25 and containing 17.5% (v/v) methanol at 1.25 ml/min. The run time was less than 10 min per sample. The eluate was analyzed for natural fluorescence (derivativization was not required) using a conventional fluorimeter (excitation wavelength 290 nm, emission wave length 400 nm). Analytical column recovery of authentic pyridinium standards was >88%. All urine samples were assayed in duplicate. Intra-assay and interassay variability were 7% and 9%, respectively.

Urine calcium (U_{Ca}) was measured by automated colorimetric assay. Urine creatinine (UCr) concentration was determined by automated analysis. Urinary hydroxyproline (Hyp) concentration was determined by colorimetry using dimethylaminobenzaldehyde. The results were all normalized to urinary creatinine excretion and expressed as nmol or μmol $mmol^{-1}$.

Pain Relief

Patients were interviewed at follow-up intervals to determine the extent of pain relief that was achieved after treatment with ^{89}Sr or EBRT. Responses were separated into complete pain relief (defined as resolution of pain requiring no analgesia or virtually no analgesia), partial pain relief (significant improvement in pain with at least a 50% reduction in analgesic requirements) and lesser degrees of pain relief.

Statistical Methods

Descriptive statistical methods were used to obtain mean, s.d. and s.e.m. data for all groups of patients. One way analysis of variance (ANOVA) including normality and equal variance testing were used to establish the significance of differences between groups of patients at an α of 0.05 or greater as indicated. The power of the performed tests was >0.85 at that α for each of the comparisons.

RESULTS

Mean 24-hr urine creatinine and calcium concentrations were within the normal range of <14 mmol and <4 mmol per day for all patients at baseline, respectively. A 15% ($p < 0.03$) and 23% ($p < 0.02$) increase after 6 mo occurred in these concentrations in the patients with metastatic prostate carcinoma in comparison to control patients, respectively. However, this comparative increase in urinary calcium excretion did not occur in the patients who received ^{89}Sr-chloride therapy. Urinary hydroxyproline concentrations remained stable over the 6-mo interval at a mean of 25 μmol $mmol^{-1}$ creatinine (normal range, 25–65 μmol $mmol^{-1}$ creatinine) (*23*). All patients maintained adequate hematological function including a platelet count of at least 100 × 10^9/liter and leukocyte count of at least 3 × 10^9/liter throughout the investigation period (6 mo).

The control group of 20 patients demonstrated mean urinary concentrations of PYD and DPD of 19.6 ± 5.6 and 6.15 ± 1.1 nmol $mmol^{-1}$ creatinine, respectively (Table 2). These findings are within the range of concentrations for healthy patients reported in the published literature (*20,24*). There was a statistically significant difference between control patients and those with mild Paget's disease or nonmetastatic (Stage A-B) prostate cancer.

Figure 2 summarizes the differences in urinary PYD concentration between patients with early stage prostate cancer and those with prostate cancer and skeletal metastatic disease. The mean PYD concentration for patients with prostate cancer was 21.7 ± 5.7 whereas the concentration of PYD in patients with osseous metastases was 30.3 ± nmol $mmol^{-1}$ creatinine, a difference of 39% ($p < 0.05$). Similarly, the concentration of urinary DPD was 10 ± 1.7 whereas patients with skeletal metastatic disease exhibited a concentration of 12.1 ± 2.4 nmol $mmol^{-1}$ creatinine, a difference of 21% ($p < 0.05$) (Fig. 3).

TABLE 2
Urinary Cross-Links in Healthy Control Patients and Prostate Cancer Patients and Patients with Paget's Disease

		PYD	DPD
	Age (yr)	nmol/mmol creatinine	
Control group (n = 20)	76.6 ± 12.2	19.6 ± 5.6	6.15 ± 1.1
Paget's disease (n = 5)	64.2 ± 7.7	29.4 ± 7.1*	10 ± 2.1*
Prostate cancer (n = 8)	76.1 ± 13.3	21.7 ± 5.7*	10 ± 1.7*

*Significantly ($p < 0.05$) different from control group.
All values are mean ± s.d.

Comparable levels of cross-links were measured in the patients who underwent ^{89}Sr-chloride or EBRT therapy before commencing such therapy (Table 3). The mean urinary concentration of PYD during the 6-mo interval after the presentation of a symptomatic osseous metastatic bone lesion is indicated in Figure 4. One group of patients received ^{89}Sr-chloride therapy as part of their management. Addition of ^{89}Sr-chloride therapy to the treatment of these patients resulted in a significantly ($p < 0.01$) lower overall production of PYD over 6 mo after ^{89}Sr-chloride administration in comparison to patients who did not receive such therapy. At 6 mo, the concentration of PYD was 62.1 ± 35.5 for the patients who did not receive ^{89}Sr-chloride therapy versus 24.6 ± 17.7 nmol $mmol^{-1}$ creatinine. This represents a difference of almost twice ($p < 0.01$) the concentration of cross-link in patients who were not treated with ^{89}Sr-chloride therapy.

The urinary concentration of DPD is similarly indicated in Figure 5. The concentration of DPD is also significantly higher over the 6-mo interval after the presentation of a symptomatic

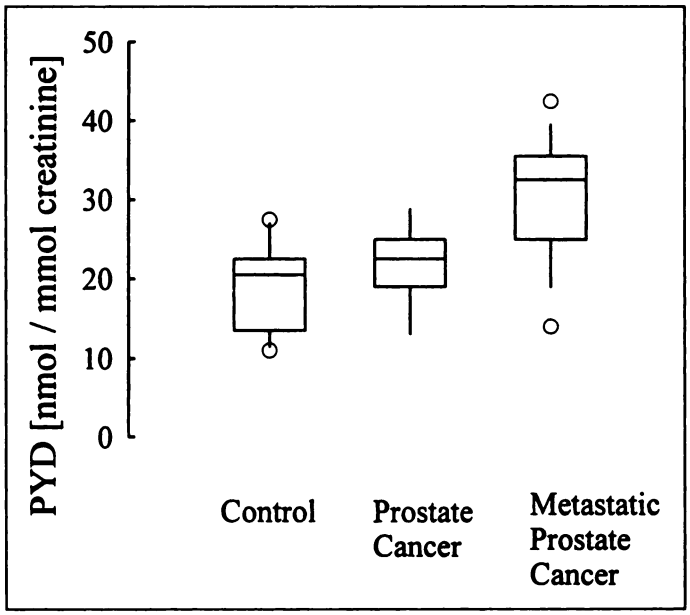

FIGURE 2. Baseline values of PYD in healthy normal (n = 20), prostate cancer (stage A-B, n = 8) and metastatic prostate cancer (n = 40) groups.

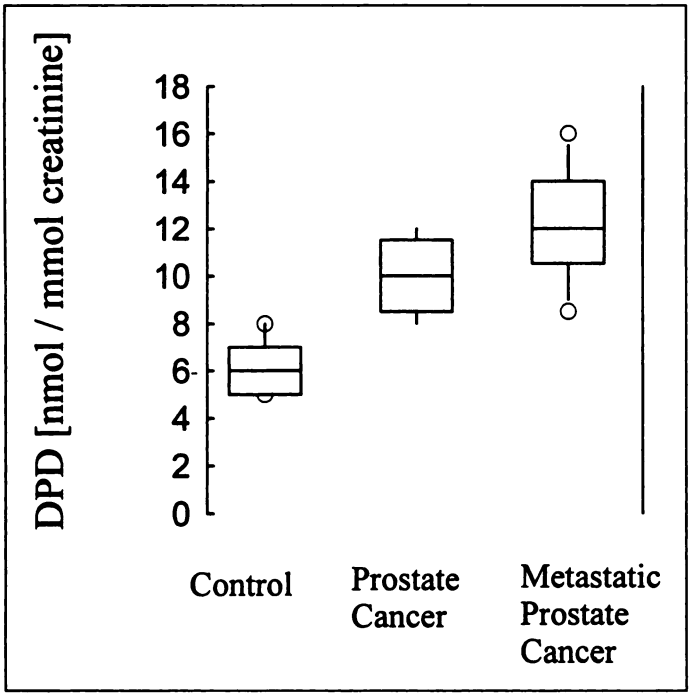

FIGURE 3. Baseline values of DPD in healthy normal, prostate cancer and metastatic prostate cancer groups.

osseous metastasis in patients who did not receive ^{89}Sr-chloride therapy versus those who did. At 6 mo, for example, the mean concentration of DPD is 55.4 ± 29 for those patients who did not receive ^{89}Sr-chloride therapy versus 14.2 ± 8.3 nmol mmol^{-1} creatinine. This represents a threefold higher urinary concentration in the patients who did not receive ^{89}Sr-chloride therapy.

Patients reported a significant level of palliation after ^{89}Sr-chloride therapy or EBRT during the follow-up interval. At 3 mo post-treatment, pain relief was reported as complete pain relief 40% for ^{89}Sr and 50% for EBRT ($p = 0.42$) and partial pain relief 40% for ^{89}Sr and 40% ($p = 0.33$) for EBRT. Similarly, no significant difference in pain response was noted at the 1- and 6-mo post-treatment intervals (data not shown). The mean cross-link concentrations for the four patients who did not respond to ^{89}Sr-chloride therapy were: PYD 52 ± 19 and 60 ± 12 nmol mmol^{-1} creatinine at 3- and 6-mo post-therapy, respectively, and DPD 15 ± 9 and 28.6 ± 11 nmol mmol^{-1} creatinine, respectively.

TABLE 3
Urinary Cross-Links in Prostate Cancer Patients with and without Skeletal Metastases

		PYD	DPD
	Age (yr)	nmol/mol creatinine	
Prostate cancer (n = 8)	76.1 ± 13.3	21.7 ± 5.7	10 ± 1.7
Metastatic prostate cancer (n = 20) (EBRT)	81.7 ± 9.5	30.3 ± 8.3*	12.1 ± 2.4*
Metastatic prostate cancer (n = 20) (^{89}Sr-chloride)	77.4 ± 9.9	31.6 ± 8.0*	12.3 ± 2.6*

*Significantly ($p < 0.05$) different from patients with no bone metastases.
All values are mean ± s.d.
EBRT = external beam radiation therapy.

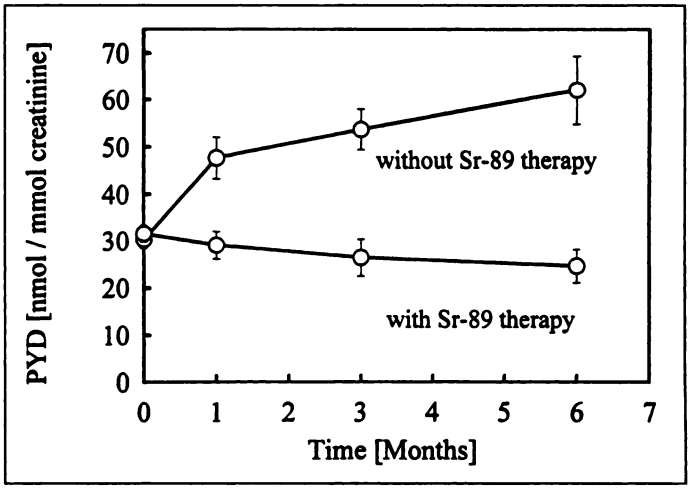

FIGURE 4. Urinary PYD concentration over a 6-mo interval following ^{89}Sr-chloride therapy or conventional palliation. Open circles represent the mean ± s.e.m. for duplicate measurements.

DISCUSSION

The data presented here demonstrate that the urinary excretion of pyridinium cross-links of collagen, PYD and DPD, serve as sensitive measures of bone resorption during ^{89}Sr-chloride therapy for metastatic prostate carcinoma. The urinary concentrations of PYD and DPD remained relatively stable in patients during a 6-mo interval after ^{89}Sr-chloride palliation. However, production of both of these cross-links increased significantly during that interval in patients with skeletal metastatic disease who did not receive ^{89}Sr-chloride therapy. Moreover, patients who did not respond to systemic treatment also demonstrated higher cross-link concentrations than the clinical responders. The reverse correlation between serum cross-link concentration and EBRT response is clearly complicated and suggests that different mechanisms may contribute to palliation in each type of therapy. It is also possible that systemic radionuclide therapy may reduce pain from micrometastases that are outside the EBRT treatment field. Consequently, these micrometastases may continue bone turnover, and they may ultimately continue to produce pain in the host.

The accurate measurement of urinary excretion of PYD and DPD is associated with factors which may significantly alter the quantitative analysis of these compounds (*15*). The use of internal standards for the assessment of recovery is crucial to accurate determination. In the present investigation, hydrolyzed elastin served as such an internal control based on literature evaluation of this compound (*15*). The recovery of this standard was comparable to published values from other laboratories. Similarly, acidification of urine samples before and during storage was also important in preserving the nascent structure of the collagen cross-links. Finally, cooling the HPLC column during separation proved invaluable in increasing the recovery yield of these compounds.

The inhibition of bone resorption reduces the release of calcium, and unchanged urinary calcium concentrations 6 mo after ^{89}Sr-chloride therapy suggest that some bone turnover may have been slowed in some patients (Figs. 4 and 5). In marked contrast, a 23% increase in urinary calcium excretion was observed after the same interval in patients who did not undergo ^{89}Sr-chloride therapy. A statistically significant decrease in urinary calcium levels has been reported after bisphosphonate treatment for metastatic skeletal disease (*25*). Diet, renal function, parathyroid hormone and parathyroid-related protein all appear to influence the measurement of urinary calcium so that it actually serves as an index of bone turnover rather than bone

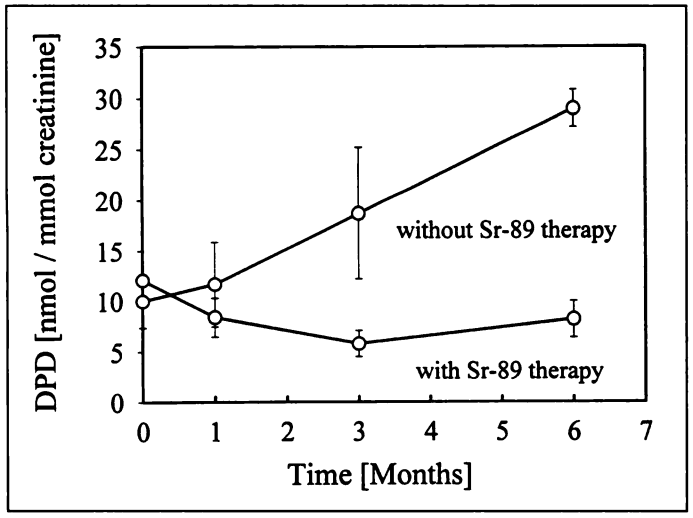

FIGURE 5. Urinary DPD concentration over 6-mo interval following ^{89}Sr-chloride therapy or conventional palliation. Open circles represent the mean ± s.e.m. for duplicate measurements.

resorption. A recent study compared 143 normal controls with 98 patients with a malignancy and identified no significant increase in urinary calcium between cancer patients with bone metastases and controls (*26*). This study did not identify any significant urinary calcium concentration differences between those two groups.

Similarly, urinary excretion of hydroxyproline is an inaccurate index of bone resorption because this imino acid is present in all collagenous tissues. Consequently, increased urinary excretion of Hyp may reflect the metabolism of any number of collagens, elastin or complement factor C1q. The concentration of Hyp remained stable during the 6-mo interval in this study despite significant changes in pryidinium collagen cross-link excretion. This suggests a dissociation between Hyp production originating from a variety of sources and bone collagen (type I)-specific production of PYD and DPD. Consequently, the relevance of urinary Hyp as an index of bone resorption is again questionable.

Recent findings regarding pamidronate for the treatment of metastatic bone resorption indicate that urinary PYD and DPD begin to decrease approximately 8 wk after treatment (*23*). The present data do not indicate a significant fall in these resorption markers, rather their production remains relatively constant during the 6 mo interval after ^{89}Sr-chloride therapy. It appears that ^{89}Sr-chloride therapy does not arrest bone resorption but instead slows the process of resorption. This is particularly evidenced by the significant increase of PYD and DPD release over the same interval in patients who did not receive ^{89}Sr-chloride therapy.

This investigation demonstrated that PYD and DPD urinary excretion increases over time in patients with metastatic prostate cancer. This observation is consistent with other reports of increased PYD and DPD in patients with neoplasms (*20–24*). Likewise, elevated levels of PYD and DPD have been reported in nonmalignant diseases associated with increased bone resorption such as Paget's disease of bone, primary hyperparathyroidism, osteoporosis, rheumatoid arthritis and osteomalacia, and were observed in this study (Paget's disease) (*27*). Strontium-89-chloride therapy significantly reduces the release of these bone resorption markers. This finding suggests that these markers may be useful objective measures of metastatic disease status.

CONCLUSION

The findings presented here demonstrate the utility of measuring urinary production of pyridinium collagen cross-links, PYD and DPD as an index of increased bone resorption in patients with prostate cancer. The measurement of these cross-links is directly related to the palliative response obtained from systemic treatment with ^{89}Sr-chloride. These bone resorption markers may be useful in determining the optimal timing for treatment or retreatment using ^{89}Sr-chloride therapy long before pain returns and scintigraphy demonstrates disease progression.

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