Urinary Excretion of Pyridinium Cross-Links of Collagen in Infancy

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This cross-sectional study evaluated urinary excretion of pyridinium cross-links of collagen, specific markers of ongoing bone resorption, in infants aged 1 week to 7 months and examined the relationship between urinary cross-links and individual renal function. Spot urines from a total of 100 infants were analyzed. The collagen cross-links, pyridinoline (Pyd) and deoxypyridinoline (D-Pyd), were assayed by fluorescence detection after high-performance liquid chromatography (HPLC). β_2-Microglobulin (β_2M), an index of renal tubular function, was determined by radioimmunoassay. In healthy term infants, urinary collagen cross-links were several times higher than the reported data for older children, with peak values seen at 1 month of age. Excretion of Pyd and D-Pyd was also markedly elevated in 1-month-old preterm infants, despite poor somatic growth. Such high excretion of collagen cross-links probably reflects the state of accelerated bone turnover in infancy. The postnatal change in the cross-links was different from that in urinary β_2M, and the values obtained did not correlate with β_2M in either term or preterm infants. These results indicate that cross-link excretion is not influenced directly by individual renal function.

THE MOST ABUNDANT protein in bone matrix is type I collagen.[1-3] The pyridinium compounds, pyridinoline (Pyd) and deoxypyridinoline (D-Pyd),[4,5] are maturation products of the lysyl oxidase–mediated cross-linking pathway of type I collagen.[1-3] When bone matrix is resorbed, the cross-linking residues, Pyd and D-Pyd, are released from the collagen molecules and eventually excreted in urine.[2,3] Urinary levels of Pyd and D-Pyd are now recognized as more specific and sensitive than urinary hydroxyproline as markers of degradation of mature collagen in bone, because urine hydroxyproline, which is derived from all types of collagen in the total body, is nonspecific.[2,3,6] Moreover, hydroxyproline is largely metabolized in the liver, and its urinary excretion, unlike pyridinium cross-links, is influenced by dietary collagen or gelatin.

Over the past several years, evidence has accumulated that quantitative measurements of these compounds in urine by high-performance liquid chromatography (HPLC) provide valid indices of bone resorption.[7-14] The HPLC-based method has been applied to assess increased pathogenic bone resorption in arthritic disease[7,12] and in a range of metabolic bone diseases such as osteoporosis,[3,9] primary hyperparathyroidism,[3,7] and metastatic bone disease.[3,10] Depression of bone turnover related to malnutrition has also been determined by this technique.[11]

In contrast to previous studies that have focused mainly on adult subjects,[3,7-10,12,13] no data are available on urinary excretion of Pyd and D-Pyd in infants born at term or preterm. Similarly, there is no information available about the effects of renal function in these infants on the cross-link markers. The purpose of this cross-sectional study was (1) to establish reference ranges of urinary collagen cross-link excretion in healthy term infants aged 1 week to 7 months, (2) to compare the cross-link excretion of preterm infants with that of term infants, and (3) to determine whether individual renal function has any influence on the urinary markers.

SUBJECTS AND METHODS

Patients

Spot urine samples were obtained between 10:00 AM and 1:00 PM from healthy term infants who came to our department for regular checkups from August 1994 to February 1995. These infants underwent ultrasound evaluation of the urinary tract, as detailed previously.[15] Only infants who had a normal urinary tract and urinalysis (ie, no proteinuria or hematuria by dipstick and no pyuria or bacteriuria using a counting chamber[16]) and no history of endocrine or metabolic disease or medication use were enrolled in the study. A total of 82 infants, all born with appropriate weight for date, were selected (Table 1). No infants were studied sequentially.

Eighteen preterm infants aged 1 month who had normal urinalysis and serum creatinine (Cr) concentrations (0.2 to 0.8 mg/dL) were also studied (Table 1). Their Apgar score (1 minute) was 6 ± 2 (range, 1 to 9). Fifteen infants had respiratory failure and required oxygen during the first 20 ± 21 days of life (range, 1 to 70), and 14 needed ventilatory support for a total of 16 ± 17 days (range, 1 to 56). All were fed their own mother's milk and/or a cow milk–based standard formula (ie, SMA-S-26, Nihon Wyeth, or Soft-Curd F&P-f, Meiji Milk Products, Tokyo, Japan). Concentrations of phosphorus, calcium, and vitamin D were 33 and 31 mg, 44 and 53 mg, and 42 and 53 IU per dL in the respective formulae.[17] Feeds were increased carefully as tolerated in each infant. The volume of feeding was 108 ± 30 (range, 52 to 155) mL/kg body weight on the study day. No infants had received supplementary amino acid and fat solutions or vitamin D medications.

All urine samples were centrifuged, and the supernatant was stored frozen at −20°C until used. Informed consent for the study was obtained from the parents of all subjects.

Reagents

Concentrated hydrochloric acid and *n*-heptafluorobutyric acid (HFBA) of amino acid analytical grade were purchased from Tokyo Kasei (Tokyo, Japan) and Wako Chemical (Osaka, Japan), respectively. All other reagents were of HPLC grade obtained from

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Table 1. Characteristics of Study Subjects

Age	No.	Sex (M/F)	Weight (g)	GA (wk)	Birthweight (g)	Feeding (n) Breast/ Formula/Mixed*
Healthy term infants						
1 wk	14	8/6	3,138 ± 218	40 ± 1	3,115 ± 234	7/2/5
1 mo	36	23/13	4,272 ± 505	40 ± 1	3,151 ± 310	12/8/16
4 mo	19	10/9	6,918 ± 863	40 ± 1	3,148 ± 371	9/3/7
7 mo	13	6/7	8,461 ± 365	40 ± 1	3,149 ± 217	3/6/4
Preterm infants						
1 mo	18	10/8	1,743 ± 644† (778-2,766)	30 ± 3 (24-32)	1,507 ± 439 (784-1,994)	3/3/12

Abbreviation: GA, gestational age.

*Type of feeding infants were receiving at the time of urine collection.

†Weight gain from birth to 1 month is significantly poorer in preterm infants as compared with 1-month-old term infants (236 ± 258 *v* 1,121 ± 372 g, $P < .001$ by unpaired *t*-test).

Wako Chemical. A standard of Pyd was donated by Dr D. Fujimoto (Tokyo University of Agriculture and Technology, Tokyo, Japan).[4,7] A mixture of Pyd and D-Pyd was provided by Dr D. Uebelhart (Inserm Unit 234 and Service de Rhumatologie et de Pathologie Osseuse, Lyon, France).[9]

Measurement of Urinary Collagen Cross-Links

Urinary Pyd and D-Pyd were assayed by HPLC with fluorometric detection according to the method of Uebelhart et al[9] with slight modifications,[18-20] and were corrected for Cr excretion. All measurements were performed in a blind fashion.

Urine extraction. One milliliter of urine was hydrolyzed with an equal volume of concentrated hydrochloric acid at 107°C for 18 hours. The cooled hydrolysate (0.25 mL) was mixed with 2.5 mL acetic acid:distilled water:*n*-butanol (1:1:8 by vol) and applied to a CF1 cellulose column (Poly-Rep column 8 × 40 mm; Bio-Rad Laboratories, Hercules, CA). After washing the column with 7 column vol buffer, the pyridinolines were eluted with distilled water (10 mL) in a glass tube and evaporated to dryness overnight. Dry residues were stored at −20°C before HPLC analysis.

HPLC assay. For HPLC analysis, the dry residue was resuspended in 500 μL 1% HFBA and centrifuged at 750 × *g* for 10 minutes to remove cellulose debris. The supernatant was collected in an HPLC vial, and 100 μL of each sample was applied to the HPLC system, which consisted of an L-6300 intelligent pump (Hitachi, Ibaragi, Japan), and F-1500 fluorescence detector using a xenon lamp with excitation and emission wavelengths of 297 nm and 395 nm, respectively (Hitachi), and an AS-2000 autosampler (Hitachi). Pyridinium cross-links were separated on a Capcell Pack C18 column (SG120, 4.6 mm × 250 mm; Shiseido, Tokyo, Japan) protected by a Capcell Pack C18 guard cartridge (4.6 mm × 50 mm; Shiseido). The column was equilibrated with HFBA: acetonitrile:sodium formate (1:5:95 by vol), and the sample was eluted with the solvent for 20 minutes at a flow rate of 1 mL/min. Eluted peaks were monitored by the F-1500 fluorescence detector and compared with those of Pyd and D-Pyd standards. After elution of each sample, the column was washed with a linear gradient of HFBA:acetonitrile:sodium formate from 1:47:53 to 1:5:95 for 7 minutes and equilibrated again in HFBA:acetonitrile: sodium formate 1:5:95 for 18 minutes at a flow rate of 1 mL/min. The overall assay variation was ±3.5% and ±7.5% for Pyd and D-Pyd, respectively. Adult normal ranges for Pyd and D-Pyd by this assay are 17.7 to 41.9 and 2.2 to 6.1 pmol/μmol Cr, respectively. Values in healthy children aged 3 to 14 years are approximately 100 to 300 and 10 to 45 pmol/μmol Cr, respectively.[19]

Measurement of Urinary Cr and β_2-Microglobulin

Urinary Cr concentration was enzymatically measured in the same sample using a Cr test kit (Creatinine HR-II Test; Wako Chemical). For most subjects, β_2-microglobulin (β_2M) was also determined using a competitive radioimmunoassay (β_2M kit II; Eiken Chemical, Tokyo, Japan) of the urine sample, which was immediately adjusted to a pH greater than 6.5 by addition of sodium azide, as reported previously.[21,22] Assay variation was ±4.4% for β_2M measurement. Urinary β_2M excretion was also expressed as ratios of the urinary Cr concentration.

Statistical Analysis

Data are presented as the mean ± SD and/or range. Statistical comparisons were made by one-way ANOVA using Scheffe's test and unpaired Student's *t*-test where appropriate. Correlation coefficients were determined by linear regression analysis. The significance threshold was retained for P less than .05.

RESULTS

Urinary Pyd and D-Pyd Excretion

Urinary excretion of total pyridinium cross-links was increased twofold to fivefold in healthy term infants as compared with the reported data for older children aged 2 to 16 years[8,13,14,19] (Fig 1 and Table 2). Both urine parameters were highest at 1 month of age, with subsequent values declining. Urine Pyd and D-Pyd were significantly correlated with each other during the observation period ($r = .95, .85, .88$, and .95 at 1 week, 1, 4, and 7 months of life, respectively; all $P < .001$). On each day of the study, there was no significant difference between males and females in either Pyd or D-Pyd (by unpaired *t*-test; data not shown).

Urinary Pyd and D-Pyd excretion rates were highly elevated in 1-month-old preterm infants, with values almost comparable to those of term infants of the same age. Urinary Cr of preterm infants was also similar to that of term infants (18.8 ± 10.4 *v* 15.4 ± 9.3 mg/dL). The correlation of Pyd and D-Pyd was again significant in preterm infants ($r = .94, P < .001$).

Urinary β_2M Excretion

In contrast to the collagen cross-links, urinary β_2M showed a peak at 1 week, with subsequent values de-

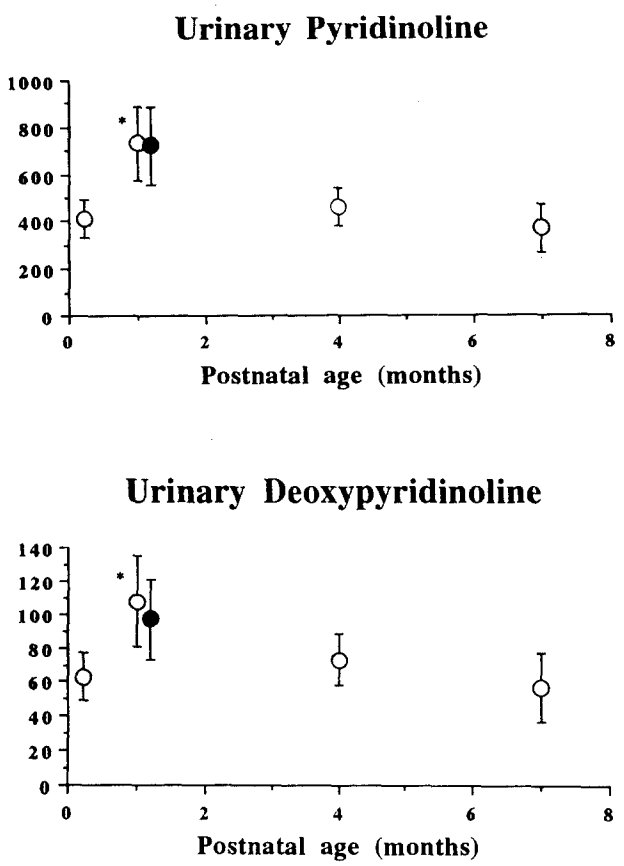

Fig 1. Variations in urinary excretion of Pyd and D-Pyd with postnatal age. Values are expressed as pmol/μmol urinary Cr and presented as the mean ± SD. (○) Healthy term infants; (●) preterm infants. *Significantly increased *v* term infants of the other age groups (*P* < .001 by Scheffe's test). There was no significant difference between 1-month-old preterm infants and term infants of the same postnatal age (by unpaired *t*-test).

clining until 4 months and thereafter staying constant in term infants (Table 2). Preterm infants excreted significantly higher amounts of β_2M in urine than their normal peers.

Relationship Between Urinary Collagen Cross-Links and β_2M

No significant correlation between urinary collagen cross-links, either Pyd or D-Pyd, and β_2M was detected in term infants at any time point in the study (Table 2). A similar lack of correlation was also found in preterm infants.

DISCUSSION

The diagnosis and treatment of a variety of metabolic bone diseases are greatly helped by the availability of specific, sensitive, and noninvasive markers of bone resorption.[3,6] Pyd and D-Pyd are cross-linking amino acids formed during maturation of fibrillar collagens.[1-3] Although small amounts of cross-links are present in other tissues such as tendons, ligaments, and the aorta, these tissues have a low turnover, making their contribution to the urinary pool small.[2,3] Thus, urinary excretion of Pyd and D-Pyd is directly related to the amount of bone resorbed and hence to bone turnover. Pyridinium compounds can be quantified precisely, using their natural fluorescence, after resolution by reversed-phase ion-pair HPLC.[7-14]

Urinary excretion of Pyd and D-Pyd in healthy individuals has been reported in several articles,[3,7,8,10,12-14] but pediatric data are still limited.[8,13,14,19] Levels of both parameters follow a characteristic pattern with age, with high values during childhood rapidly decreasing afterward and stabilizing at low levels in adulthood. In healthy children, excretion of Pyd and D-Pyd is two to 10 times greater than in normal adults, most likely a reflection of increased turnover of the growing skeleton.

In this study, we evaluated on a cross-sectional basis the excretion rates of Pyd and D-Pyd in term infants aged 1 week to 7 months, since no data for this age group are available despite the fact that infancy is the period of fastest somatic and skeletal growth. In our infants, urinary Pyd and D-Pyd levels were two to five times higher as compared with levels in older children,[8,13,14,19] which appears consistent with a recent study using an immunoassay for cross-linked *N*-telopeptides of collagen type I.[23] High levels of the urine cross-links in these infants would reflect the state of most-increased bone resorption concomitant with progres-

Table 2. Urinary Excretion of Collagen Cross-Links and β_2M

	Pyd (pmol/μmol Cr)	D-Pyd (pmol/μmol Cr)	β_2M (mg/g Cr)	Correlation Coefficient (*r*)*	
				Pyd *v* β_2M	D-Pyd *v* β_2M
Healthy term infants					
1 wk	411 ± 81	63 ± 14	12.3 ± 11.4†	−.24	−.03
	(290-520)	(39-79)	(0.65-33.7) [n = 13]		
1 mo	730 ± 157‡	108 ± 27‡	4.16 ± 4.21	.01	.04
	(449-1,070)	(57-172)	(0.94-15.5) [n = 30]		
4 mo	462 ± 80	73 ± 15	0.54 ± 0.21	−.09	−.21
	(333-660)	(50-109)	(0.29-1.05) [n = 16]		
7 mo	368 ± 98	57 ± 20	0.61 ± 0.19	.06	.11
	(201-618)	(28-111)	(0.30-1.02) [n = 11]		
Preterm infants					
1 mo	723 ± 167	97 ± 24	23.9 ± 19.9§	−.32	−.45
	(430-1,033)	(51-147)	(2.99-62.5) [n = 18]		

*No significant correlation between Pyd and β_2M or D-Pyd and β_2M in either term or preterm infants.

†P < .001 *v* the other age groups (by Scheffe's test).

‡P < .001 *v* the other age groups (by Scheffe's test).

§P < .001 *v* 1-month-old term infants (by unpaired *t*-test).

sive bone modeling. The biology underlying bone growth and modeling requires an increase in bone resorption (and hence in urine collagen cross-link excretion), as well as in bone formation. In this regard, elevated levels of serum osteocalcin, a biomarker of osteoblastic activity, are observed in infants.[24]

Details on postnatal age-related changes in the urine markers were obtained from this study. We found that urinary pyridinium compounds reached peak levels at 1 month of age. This finding is not surprising, given that the most accelerated growth and presumably bone turnover take place at around this age. No significant sex variation of cross-link excretion was seen. However, our study population was small, and individual infants were not fed in the same way (Table 1). More subjects are needed to resolve this issue.

Clinical applications of the pyridinium markers should include the osteopenia of prematurity, since this condition is frequent in preterm infants.[17,25-27] It is characterized by an imbalance between bone resorption and formation associated with poor bone mineralization or bone loss after birth. The 1-month-old preterm infants we studied showed a high excretion of Pyd and D-Pyd in urine almost comparable to that observed in term infants of the same postnatal age, although the somatic (and probably skeletal) growth of preterm babies was poor as compared with term infants (Table 1). Our previous radiologic[17] and current biochemical findings appear consistent with the state of "high-turnover" osteopenia in preterm babies.[27] Longitudinal studies combined with bone mineral assessments would be necessary to further elucidate the osteopenia of prematurity.

We also examined the relationship between urinary cross-link excretion and renal function in our subjects. Infantile kidney function is still immature (even in term infants) and may be aggravated in various disease states such as premature birth, asphyxia, and respiratory distress.[21,22,28] Our preterm infants excreted significantly higher amounts of β_2M, a sensitive marker of renal tubular function,[21,22,28] in urine as compared with term infants, indicating that they had more immature and/or damaged kidneys at 1 month of age. We initially hypothesized that the degree of individual renal function of the infants might contribute to the urinary excretion of pyridinium cross-links, as found in the excretion of growth hormone.[29,30]

Unexpectedly, the postnatal change in urine cross-links is different from that of β_2M; β_2M excretion is highest around 1 week of age in healthy term infants, as demonstrated by our previous studies[21,22] and by the present study. The pattern of change also differs from that of urine albumin, a marker of glomerular sieving function, since urinary albumin excretion gradually decreases after birth.[22] Moreover, no significant correlation was detected between cross-link excretion and β_2M in either term or preterm infants. These observations support the conclusion that excretion of pyridinium compounds is not influenced directly by infantile renal function. Along the same line, McLaren et al[12] recently showed that in adult patients with chronic arthritis and renal impairment, cross-link excretion rates are not related to the degree of impairment at either the renal glomerular level (assessed by Cr clearance rate) or the tubular level (assessed by urine *N*-acetyl-β-D-glucosaminidase activity). These findings are certainly relevant to the potential use of cross-link markers for infants with kidney disease.

In conclusion, this study shows that urinary excretion of the collagen cross-links, Pyd and D-Pyd, is elevated in infants born at term or preterm, and that the excretion is not related directly to individual renal function, but probably reflects the state of accelerated bone turnover in infancy. With the normal values established here, these markers can now be used for diagnosis and follow-up evaluation of metabolic bone diseases and for monitoring therapeutic treatment in infants.

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