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Urinary Pyridinoline Cross-Links in Ehlers-Danlos Syndrome Type VI

To the Editor:

The Ehlers-Danlos syndrome (EDS) is a heterogeneous group of heritable disorders of connective tissue, affecting skin, ligaments, joints, blood vessels, and internal organs. The main general findings are hyperextensibility and bruisability of the skin, with abnormal scarring, and joint laxity. On the basis of clinical, genetic, and biochemical findings, EDS can be classified today into at least 10 different types (Steinmann et al. 1993). Among them, EDS type VI (MIM 225400) is characterized by marked muscular hypotonia from birth; kyphoscoliosis, often present at birth and progressing to a severe form; marfanoid habitus; eye involvement, often with microcornea and a tendency of the eyeballs to rupture after minor trauma; osteoporosis; and sometimes spontaneous rupture of arteries. The disorder is due to a deficiency of lysyl hydroxylase (E.C.1.14.11.4), inherited in an autosomal recessive mode. Traditionally, the clinical diagnosis is confirmed by an insufficiency of hydroxylysine, on analysis of hydrolyzed dermis and/or reduced enzyme activity in cultured skin fibroblasts (for review, see Steinmann et al. 1993). We present data on urinary excretion of lysyl- and hydroxylysyl-derived pyridinoline cross-links in seven genetically defined patients with EDS type VI and confirm and ex-

Table 1

Urinary Excretion of Pyridinoline Cross-Links in Seven Patients with EDS Type VI

Subject(s)[a]	Sex	Age (years)	Genotype[b]	HP[c] (nmol bone collagen/ mmol creatinine)	LP[c] (nmol bone collagen/ mmol creatinine)	HP + LP[c] (nmol bone collagen/ mmol creatinine)	LP:HP	NTx[c]
Patient 1○	M	3	dup/?	53	277	330	5.3	255
Patient 2○	M	8	dup/?	80	477	577	5.9	382
Patient 3●	F	9	dup/dup	150	764	914	5.1	377
Patient 4●	M	13	dup/dup	87	517	604	5.9	404
Patient 5□	M	13	dup/dup	127	721	848	5.7	628
Patient 6■	F	13	stop/stop	12	79	91	6.8	40
Patient 7■	F	18	stop/stop	17	99	116	6.0	61
Parent of 5□	F	32	dup/+	28	17	45	.6	20
Parent of 5□	M	42	dup/+	18	6	24	.3	21
Control children[d] (n = 12)		2–15		372 ± 74	106 ± 21	478 ± 95	.29 ± .02	629 ± 190[e]
Control adults[d] (n = 37)		21–70		27 ± 2	9 ± 1	36 ± 3	.30 ± .02	37 ± 13[f]

[a] Individuals having the same superscript symbol (unblackened circle, black circle, unblackened square, or black square) are members of the same family.

[b] "dup/dup" and "dup/?" denote the homozygous and the compound heterozygous state, respectively, for a large duplication (unpublished), in the gene for lysyl hydroxylase, identical to that described by Hautala et al. (1993), but in different patients; and "stop/stop" denotes a homozygous stop codon in the gene, as described by Hyland et al. (1992).

[c] Means of duplicates.

[d] From Beadsworth et al. (1990).

[e] From Bollen and Eyre (1994); mean ± 1 SD (range 47–2,430; n = 870).

[f] From Hanson et al. (1992); mean ± 1 SD (range 10–89; n = 50; age range 24–40 years).

tend previously published results (Pasquali et al. 1994).

Lysyl hydroxylase hydroxylates lysyl residues on nascent intracellular procollagen α-chains, and its impairment results in a low hydroxylysine content in mature triple-helical collagen molecules. After secretion and lysyl oxidase–mediated oxidation of specific lysyl and hydroxylysyl residues, adjacent collagen molecules interact to form pyridinoline cross-links. Collagen degradation results in two forms of stable pyridinoline cross-links in urine: hydroxylysyl pyridinoline (HP), a more abundant component derived from three hydroxylysine residues; and lysyl pyridinoline (LP), normally less abundant and derived from one lysine and two hydroxylysine residues. Bone is believed to be a primary (but not the only) source of these cross-links in urine (Robins et al. 1991).

The diagnosis of EDS type VI was established in all seven patients, on clinical grounds, and was confirmed by a marked reduction of dermal hydroxylysine content (patients 1–7), by a marked reduction of enzyme activity (patients 5–7), and by molecular studies (patients 1–7; see table 1). Spot urine samples were obtained from the subjects at random times and were frozen without preservatives, protected against light, lyophilized, and shipped to the laboratory, where they were reconstituted with water and were analyzed. The pyridinoline cross-links (i.e., HP and LP) were recovered from acid hydrolysates of urine by gel filtration and were analyzed by reverse-phase high-performance liquid chromatography using a fluorescence detector (Eyre 1987); the interassay coefficients of variation were 6% and 9% for HP and LP, respectively. Urinary levels of collagen I cross-linked N-telopeptides (NTx) were measured by use of a novel immunoassay that specifically recognizes certain trivalent cross-linked peptides formed between collagen I N-telopeptides and a lysine or hydroxylysine residue from a collagen triple-helical domain (Hanson et al. 1992). These peptides are terminal degradation products of a specific trivalent cross-linking site in collagen I, which is particularly abundant in bone. The interassay coefficient of variation was 7% (Bollen and Eyre 1994).

Our data (table 1) show that the LP:HP ratios for all patients are in the same narrow, abnormally high range, comparable with those reported elsewhere (Pasquali et al. 1994) and in contrast with the normal ratio for the two (heterozygous) parents. For the patients, the mean urinary excretion of HP is significantly reduced, and mean LP excretion is increased several fold, apparently in proportion to the decrease in HP. The possibility that the underhydroxylated col-

lagen in bone might be turned over faster was investigated by use of the NTx assay, which is a more specific marker for bone resorption than is total pyridinoline and for which extensive age-matched control data are available (Hanson et al. 1992; Bollen and Eyre 1994; Garnero et al. 1994). This immunoassay is independent of the nature of the trivalent cross-link (e.g., both HP- and LP-containing peptide forms are equally recognized). The results (table 1) showed a strong correlation between NTx and the sum of HP and LP (r^2 = .87) but no evidence, from comparison with normal ranges, of a dramatic bone-resorption increase to explain the osteopenia observed consistently in all patients with EDS VI (Steinmann et al. 1993); however, the considerable bone-resorption variability, observed between individual children, that was dependent on their growth status (Bollen and Eyre 1994) prevents a firm conclusion on this point.

Patients 1–5, who are either homozygous for the duplication or compound heterozygous for the duplication and a yet unidentified mutation, excrete higher total pyridinolines than do patients 6 and 7. This may be because there was some residual enzyme activity in patients 1–5 or because neither patient 6 nor patient 7 was in an active growth phase. Because the assay for lysyl hydroxylase is not very sensitive (Hyland et al. 1992; Steinmann et al. 1993), urinary pyridinoline differences between the two groups of patients cannot reliably be attributed to differences in measured residual activity. More EDS patients with defined mutations in the lysyl hydroxylase gene need to be studied before it can be concluded that the lower total pyridinoline excretion in patients 6 and 7 reflects a distinct disease pattern for their mutation. This mutation, a homozygous stop codon in position 319, eliminates the enzyme's highly conserved C-terminal half, believed to be responsible for the binding of the ferrousion cofactor, and thus should result in a complete lack of activity (Hyland et al. 1992). Therefore, at present, it is unknown how, in these siblings, pyridinoline cross-links are formed in the absence of lysyl hydroxylase. Indeed, not only is LP formed, which requires hydroxylysine in telopeptides, but also some HP, which requires hydroxylysine within the triple helix as well. The most plausible explanation for pyridinoline formation by the two sibling patients is the existence of the predicted but still hypothetical N-telopeptide–specific lysyl hydroxylase (Royce and Barnes 1985; Gerriets et al. 1993). Furthermore, the results imply an alternative source of hydroxylase activity for at least some triple-helical sites of lysine in collagen, as well as the telopeptide hydroxylase activity. We conclude that the determination of urinary pyridinoline cross-links is a noninvasive, simple, and indeed reliable diagnostic test for EDS type VI.

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Pyridinium Cross-Links in Heritable Disorders of Collagen

To the Editor:

Ehlers-Danlos syndrome (EDS) is a heterogeneous group of inherited disorders of collagen that is characterized by skin fragility, skin hyperextensibility, and joint hypermobility (Byers 1995). EDS type VI is caused by impaired collagen lysyl hydroxylase (procollagen-lysine, 2-oxoglutarate 5-dioxygenase; E.C.1.14.11.4), the ascorbate-dependent enzyme that hydroxylates lysyl residues on collagen neopeptides (Krane et al. 1972; Pinnell et al. 1972; Elsas et al. 1978). Different alterations in the gene for collagen lysyl hydroxylase have been reported in families with EDS type VI (Hautala et al. 1993; Ha et al. 1994). In EDS type VI, impairment of collagen lysyl hydroxylase results in a low hydroxylysine content in mature collagen. Hydroxylysine is a precursor of the stable, covalent, intermolecular cross-links of collagen, pyridinoline (Pyr), and deoxypyridinoline (Dpyr). Elsewhere we reported in preliminary form that patients with EDS type VI had a distinctive alteration in the urinary excretion of Pyr and Dpyr (Pasquali et al. 1994). In the present study, we confirm that the increased Dpyr/Pyr ratio is specific for EDS type VI and is not observed in other inherited or acquired collagen disorders. In addition, we find that skin from patients with EDS type VI has reduced Pyr and increased Dpyr, which could account for the organ pathology.

Cross-linking of lysyl and hydroxylysyl residues occurs after processing of procollagen and secretion of the collagen molecule into the extracellular space (Byers 1995). Specific lysyl or hydroxylysyl residues in the telopeptide regions at both ends of the collagen molecule are oxidized in the extracellular space to form aldehydes (Eyre et al. 1984*b*) by the copper-dependent enzyme lysyl oxidase (protein lysyl 6-oxidase; E.C.1.4.3.13). These aldehydes react with specific peptidyl lysyl or hydroxylysyl residues in the triple helical domain on juxtaposed neighboring molecules in a fibril to form bifunctional reducible intermediates that mature into trifunctional, nonreducible pyridinium cross-links (Eyre et al. 1984*b*). Two major forms have been identified: Pyr, derived from three hydroxylysyl residues, and Dpyr, derived from one lysyl and two hydroxylysyl residues. Pyr and Dpyr are abundant in hard and load-bearing tissues, such as bone, cartilage, and dentin (Eyre et al. 1984*a*). Skin and other soft connective tissues are thought to lack pyridinium cross-links (Eyre et al. 1984*a*). Both Pyr and Dpyr are biologically and chemically stable and are excreted in urine in free and peptide-bound forms as products of collagen degradation. Their urinary excretion correlates with bone turnover in acquired and inherited disorders affecting bone (Beardsworth et al. 1990; Uebelhart et al. 1990; Paterson et al. 1991; Robins et al. 1991; Coleman et al. 1992; Gay et al. 1994).

We analyzed urine (first void morning urine) from six patients with EDS type VI whose diagnoses were established by history, clinical evaluation, quantitation of the total hydroxylysyl residues in skin collagen, and assay of collagen lysyl hydroxylase in cultured dermal fibroblasts using nonhydroxylated, tritiated chick embryo bone procollagen as substrate (Elsas et al. 1978; Miller et al. 1979; Dembure et al. 1984, 1987). Enzyme activity was determined in fibroblasts by using methods described elsewhere (Miller et al. 1979). The mutations in the lysyl hydroxylase gene have been identified in one of the patients studied (Ha et al. 1994). Other collagen and bone disorders were clinically diagnosed and confirmed by laboratory and radiographic procedures. Pyridinium cross-links were extracted from urine hydrolysates and analyzed by high-performance liquid chromatography according to established procedures (Uebelhart et al. 1990). Pyridinium cross-link concentration was normalized to urinary creatinine. Creatinine was quantified using a Beckman Creatinine Analyzer 2.

Skin biopsy material was obtained from patients and controls with informed consent approved by the Emory University School of Medicine internal review board. Pyridinium cross-links and amino acids in skin were expressed as mol/mol of collagen. Collagen was estimated based on the amount of hydroxyproline, assuming that collagen weighed 7.5 times the measured hydroxyproline weight and had a molecular weight of 300,000 (Eyre et al. 1984*a*).

Table 1 reports the urinary excretion of pyridinium cross-links in patients with inherited bone and collagen disorders and in age-matched controls. In controls, the urinary excretion of pyridinium cross-links was higher in infancy and childhood, reflecting rapid bone growth and remodeling (Beardsworth et al 1990). Despite a decrease with age of total pyridinium cross-link excretion, the Dpyr/Pyr ratio did not change significantly (range 0.21–0.28). Patients with EDS type VI had decreased Pyr and increased Dpyr urinary excretion, with a significant increase in the Dpyr/Pyr ratio (range 4.87–7.09). This change in the Dpyr/Pyr ratio was not ob-