The specificity of collagen cross-links as markers of bone and connective tissue degradation

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The availability of a specific and convenient biochemical assay for quantifying bone turnover would aid in the clinical management of osteoporosis and other metabolic bone diseases. Recently, several immunoassays for bone metabolites have been reported in the research literature, with data suggesting that certain of them may now have the required specificity and responsiveness to bone cell activity and convenience for routine clinical use.

Biochemical assays for monitoring bone metabolism usually target enzymes or other proteins synthesized by osteoblasts or osteoclasts that can be found in serum, or degradation products of the organic fabric of the resorbed bone that are excreted in urine (1,2). The most convincing current markers of bone formation are serum levels of skeletal alkaline phosphatase and osteocalcin. These markers usually show a lag period of several months in responding to natural and therapeutically induced changes in bone turnover rate (3), consistent with the concept that formation is coupled to but lags behind resorption at remodeling sites. Most bone resorption markers, in contrast, rely on the measurement of metabolites of collagen generated by osteoclasts in the process of degrading bone. Urinary hydroxyproline is a traditional indicator, but newer and more convenient immunoassays have focused on collagen cross-linking metabolites which offer greater specificity to bone collagen than hydroxyproline.

Pyridinolines

The pyridinoline cross-linking amino acids of collagen have received much attention as bone resorption markers in urine (4, 5). Two forms exist, the more abundant hydroxylysyl pyridinoline (HP or pyridinoline) and lysyl pyridinoline (LP or deoxypyridinoline). These trivalent structures form the mature cross-linking residues in polymers of several collagen types (collagens I, II, III and IX all use this mechanism; 6) with considerable variation in concentrations between tissues. Most connective tissues contain pyridinolines, including bone, cartilage, blood vessels, fascia, ligament, intestine, muscle, liver, indeed essentially all internal connective tissues. Skin, sclera and cornea are the notable exceptions (6). Table 1 summarizes data on the pyridinoline content of the major connective tissues of joints. Bone collagen is the primary repository of LP (deoxypyridinoline) in the body and has the highest LP:HP ratio at about 0.3:1 in humans, but other tissues, for example vascular tissue, equal bone in LP concentration when expressed as moles LP/mole of collagen (7). Because bone is remodeled faster than other major connective tissues, the pyridinolines, and deoxypyridinoline in particular, have proven in research studies to be useful urinary indicators of bone resorption activity. Their degree of specificity to bone, however, is still debatable. There is mounting evidence, particularly under certain physiological and disease conditions, that a significant fraction in urine, even of LP, is derived from tissues other than bone.

As an example, Figure 1 shows HPLC analyses of pyridinolines in aorta and muscle collagens. Although the HP:LP ratio at 8:1 for aorta and 6 to 7:1 for skeletal muscle are about double that of bone, the LP concentrations in moles/mole of collagen are all similar. Little is known about the turnover rates of collagen in blood vessels or skeletal muscle in adult humans, though skeletal muscle as an organ is known to remodel actively throughout life and muscle mass falls with increasing adult age. The contribution of these and other soft tissues to urinary levels of the pyridinolines, including LP, may, therefore, be significant and explain in part why free and total pyridinolines are proving to be less specific bone resorption markers than previously anticipated.

The following simple calculations illustrate the point. Given that the HP:LP ratio in human bone is

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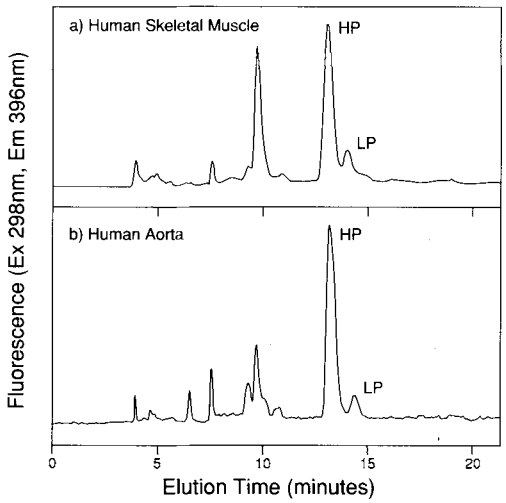

Figure 1. Reverse-phase HPLC of pyridinoline cross-links in acid hydrolysates of a) human skeletal muscle, b) human aorta. The ratios of hydroxylysyl pyridinoline (HP) to lysyl pyridinoline (LP) are 6.5:1 and 8:1, respectively.

Table 1. Distribution of pyridinoline cross-links in human bone and joint tissues in moles/mole collagen ±SD

	n	HP	LP	HP:LP ratio	Ref
Bone, cortical	18	0.26±0.09	0.07±0.02	3.5:1	23
, cancellous	18	0.18±0.08	0.05±0.02	3.5:1	23
Articular cartilage	18	1.48±0.32	<0.03	>40:1	23
Meniscus	24	1.40±0.14	<0.05	>20:1	–
Capsule	14	0.86±0.17	<0.07	>12:1	–
Annulus fibrosus	6	1.66±0.14	<0.03	>50:1	–
Nucleus pulposus	21	1.59±0.44	<0.03	>50:1	–

– Unpublished

3.5:1 (8, Table 1) and in human muscle is 7:1 (Figure 1), then if the total urinary pool of HP plus LP comes equally from muscle and bone, the resulting urinary HP:LP ratio would be 4.8:1. In other words, half the total urinary pyridinolines could come from a non-bone source and still give an HP:LP ratio that is typical of urine. The normal range for urine varies from 3:1 to 7:1 between subjects and reporting laboratories (8). In the above example, 64% of the LP and 47% of the HP would be derived from bone.

Until recently, most clinical studies have reported total pyridinolines in urine measured by HPLC after acid hydrolysis to convert peptides to the free amino acids. (The ratio of peptide to free forms in urine is usually about 2:1). However, an immunoassay for free pyridinoline was reported (9), and two commercial microtiter-plate assays based on this concept are available (Metra Biosystems Pyrilinks™ which recognizes both structures (9, 10) and PyrilinksD™ which is specific for deoxypyridinoline, i.e., LP). In a different approach, assays recognizing small cross-linked peptides that are derived from type I collagen have been introduced (Ostex's Osteomark® assay for cross-linked N-telopeptides (NTx) and Osteometer's CrossLaps™ assay for the C-telopeptide cross-linking domain). A serum assay (ICTP) designed to measure a cross-linked C-telopeptide domain of bone type I collagen (11) was also introduced by Orion Diagnostica. The obvious advantage of such immunoassays is their convenience, requiring no pre-treatment of urine, and standard ELISA methodology. The critical question is their degree of specificity to bone resorption. Theoretical biochemical advantages and disadvantages of measuring total pyridinolines, free pyridinolines, deoxypyridinoline alone and cross-linked peptides can be debated, but the proof of bone specificity eventually must rest on clinical studies.

Cross-linked telopeptide assays

Figure 2 illustrates the two locations of pyridinoline cross-links in type I collagen, N-telopeptide-to-helix and C-telopeptide-to-helix. Peptides from both sites can be recovered from urine (12). The HP:LP ratio of the cross-linked N-telopeptides in urine indicates an origin in bone. Analysis of bone collagen showed that two-thirds of the LP in bone collagen is located at the N-telopeptide site and one-third at the C-telopeptide site (12). The α2(I) N-telopeptide is also heavily involved in pyridinoline cross-linking at this site in bone collagen, apparently a distinctive feature over non-mineralized collagens. Cross-linked N-telopep-

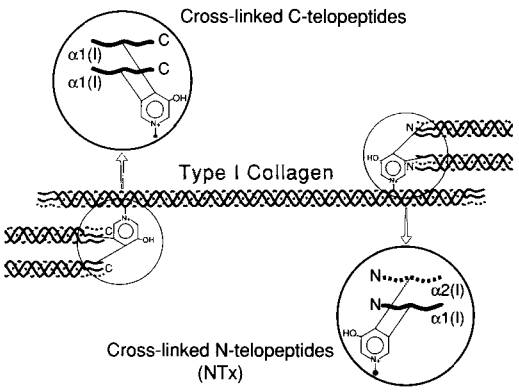

Figure 2. Sites of pyridinoline cross-linking in type I collagen fibrils. In bone, the N-telopeptide pyridinolines favor the incorporation of α2(I) over α1(I)-telopeptides.

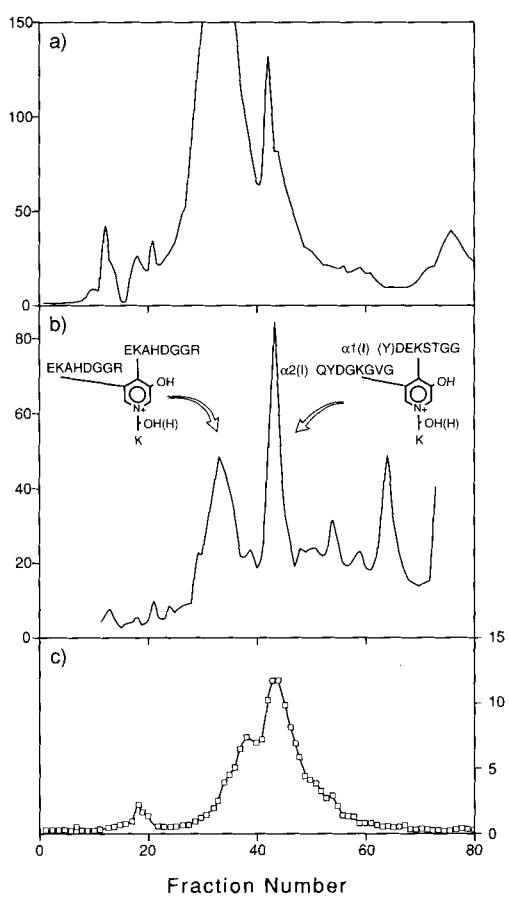

Figure 3. Molecular sieve chromatography of pyridinoline-containing peptides from urine. The peptide pool extracted from a child's urine was eluted from a Bio-Gel P10 column in 10% (v/v) acetic acid (12) monitoring fractions for pyridinoline fluorescence. The suppressed fluorescence of the N-telopeptide pool (a) is revealed after x20 dilution in 50% (v/v) acetonitrile (b). The mAb 1H11 was used in a competition ELISA format (12) to locate the cross-linked N-telopeptides (c).

tides in urine, therefore, were targeted as a particularly promising marker of bone resorption (12). A monoclonal antibody, mAb 1H11, was raised which recognized cross-linked N-telopeptides containing the α2(I) N-telopeptide sequence QYDGKGVG, where K is involved in trivalent cross-linking (12). The antibody does not recognize synthetic linear sequences matching the individual telopeptides, nor is the pyridinoline cross-linking structure itself part of the epitope (12). Moreover, the peptide antigen, NTx, becomes recognizable by the antibody only when bone collagen is degraded to small peptides, for example by osteoclasts which have been shown to generate NTx (but not free pyridinolines) when cultured on human bone particles in vitro (13).

Figure 3 shows the resolution of the two main pools of pyridinoline cross-linked telopeptides in urine by gel filtration. Pyridinoline fluorescence is quenched in the N-telopeptide structures in aqueous solvent, compared with the cross-linked C-telopeptides (panel a) as revealed by dilution in organic solvent (panel b). This may explain why others have failed to detect significant levels of pyridinoline cross-linked N-telopeptides in bone collagen (22).

Immunoreactive NTx peptides can also be quantitatively and reproducibly generated from decalcified human bone by exhaustive digestion with bacterial collagenase (12). Other tissues (e.g., skin, tendon, etc.) that contain type I collagen will also produce immunoreactive peptides by such treatment in vitro, but in significantly lower yields than bone (unpublished observations). Whether soft tissues generate immunoreactive NTx in vivo that survives into urine is unknown. In contrast, it is not clear where in the body the pool of free pyridinolines found in urine is generated, and in particular whether bone collagen is the only source. Reported ratios of pyridinoline to deoxypyridinoline in urine suggest that bone is the primary source, but not the only one. Indeed, recent reports showing a lack of suppression of urinary free pyridinolines measured by the Pyrilinks™ assay in a clinical trial of the antiresorptive bisphosphonate, alendronate (14) and other studies of bisphosphonate therapies (17), raise questions about the specificity of this analyte to the osteoclastic resorption process. Similarly, a short-term comparative study in males showed a lack of responsiveness of urinary free pyridinolines to bisphosphonate suppression of bone resorption, in contrast to NTx levels which fell 85% from baseline (15). The latter study also compared the effect on these markers of thyroid hormone (T3). The results demonstrate that NTx is a more selective index of bone resorption activity than free pyridinolines. The latter appear to respond to general changes in body metabolism, for example as stimulated by thyroid hormone.

The above report (14), comparing biochemical markers of bone metabolism, also found no increase in serum ICTP in women post-menopausally or suppression on bisphosphonate therapy, indicating that this assay does not provide an index of bone resorption rate.

The CrossLaps™ assay uses a polyclonal antiserum raised against a synthetic octapeptide (16) corresponding to the fragment of the C-telopeptide domain of the α1(I) chain of type I collagen known to be excreted in urine embodied in pyridinoline cross-

linked peptides (12; Figure 3). This sequence, EKAHDG-G-R, was chosen (16) because of its known involvement in cross-linking and the proposed protection of cross-linked telopeptides by the proximity of the cross-linking residue (12). The specificity of this assay to bone resorption will depend on the degree to which tissues other than bone contribute degraded type I collagen to urine, including whether immunoreactive linear sequences of aborted type I collagen biosynthesis are also represented. Again, a critical clinical test of the specificity to bone resorption of all such assays will be their responsiveness in human subjects to antiresorptive therapies, particularly the more advanced generation of bisphosphonates which are believed to target osteoclasts highly selectively. Another important question is whether elevation of such markers in hyperthyroidism is bone specific or reflects more a general stimulation of collagen metabolism. The published data on free pyridinolines (15, 17) and the cross-linked telopeptide EKAHDGGR (CrossLaps™) (17), for example, show greater elevation than NTx in hyperthyroidism, suggesting contributions from other tissue sources.

In summary, from the body of published clinical data (12, 14, 15, 17, 18, 19, 21), the NTx assay appears to provide the most specific and responsive biochemical index of bone resorption activity available. This may be explained (Figure 4) if the peptide analyte, NTx, is a direct and relatively unique product of osteoclastic degradation of bone, is rapidly cleared by the kidney and so acts as an indicator of recent, collective osteoclast activity in the body. Total and free pyridinolines may be less specific if they are produced by tissues in addition to bone, and, in the case of free pyridinolines, involve a non-osteoclastic pathway. The potential clinical value of a bone-specific resorption marker is in monitoring the response of individual patients to anti-resorptive therapies (estrogen, calcitonin, bisphosphonates), and thereby assuring compliance, that the dosage is effective and continues to work as a preventive therapy for rapid bone loss. As a diagnostic screening aid, an accurate resorption index might also be used to identify postmenopausal subjects who are fast losers. In conjunction with bone densitometry to identify those who also have low bone mass, this strategy could target the group with the highest risk of future osteoporotic fracture for hormone replacement or other therapies. The great advantage of a resorption marker over a bone formation marker is the rapid biological response, for example to changing estrogen status at menopause and to therapeutic interventions that target osteoclast activity as their key mode of action.

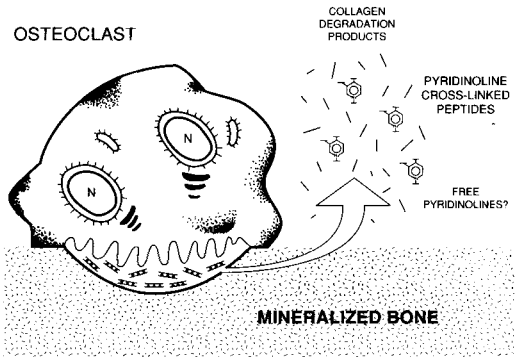

Figure 4. Resorption of bone collagen by the osteoclast. Collagen degradation products, including pyridinoline-containing peptides end up in the blood and are excreted by the kidney. The roles of the liver and kidney in further processing and the site of free pyridinoline production are unknown.

Acknowledgments

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