Collagen crosslinks in metabolic bone disease

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Noninvasive techniques for monitoring bone resorption have received a great deal of research interest in recent years, driven partly by the increasing incidence of osteoporotic bone fractures, with their attendant social and economic costs. Demographic changes suggest that osteoporosis is likely to become an increasing problem but there are now also an increasing number of treatment strategies for the disease. Suitable means of measuring bone resorption rates are therefore important not only as an aid to detecting those at risk but also for effective monitoring of therapy. These arguments, particularly in terms of monitoring therapy, are equally important for many other metabolic bone diseases.

The development of new markers for bone resorption has been an area of intense research interest over the past few years and there have been a number of recent developments in techniques. The aim here is to describe briefly these new techniques and to consider their relative merits in comparison with existing methods.

Rationale for using collagen crosslinks

Until recently, the main method available clinically for the assessment of bone resorption was urinary hydroxyproline. It was recognised, however, that this method lacked specificity and sensitivity for a number of reasons (Robins 1982a) and in considering the new methods that have recently been developed, it is essential to keep in mind the characteristics required for a valid assay and how well the drawbacks inherent in hydroxyproline measurements are overcome. A major problem with measuring hydroxyproline is presence of this imino acid in all connective tissues as well as some rapidly metabolised serum components such as C1q. Use of the collagen crosslinks, pyridinoline (Pyd) and deoxypyridinoline (Dpd) was a major step in providing tissue specificity because of the restricted distribution of these crosslinks (Robins et al. 1990, Seibel et al. 1992). In particular, both crosslinks are absent from normal skin except for the very small amounts that may be present in blood vessels (Reiser et al. 1987). Pyd has a wider tissue distribution than its deoxy analogue, is present in highest concentrations in cartilage but also constitutes one of the major crosslinks of bone collagen. By contrast, Dpd is present mainly in bone collagen in which the ratio Pyd/Dpd is generally about 4:1 (Eyre et al. 1988), similar to that determined in adult urine (Robins et al. 1991a). These observations suggest that bone resorption is the major contributor of both crosslinks in urine, a view strengthened by the close concordance between urinary concentrations of Pyd and Dpd in most circumstances. Only in patients with active rheumatoid arthritis has some disparity between the two crosslinks been observed, probably resulting from an additional contribution of Pyd from joint tissue turnover (Seibel et al. 1989). Dpd is present also in aorta and some other blood vessels as well as in ligaments (Robins et al. 1990) but body composition studies in sheep have shown that the contribution from other tissues is very low (Scott et al. 1993); in addition, all available evidence suggests that the metabolic turnover rate of these tissues is very slow (Robins et al. 1982a). Consequently, in terms of measuring the compound in body fluids, Dpd is essentially a bone-specific marker and represents the most bone-specific resorption marker currently available.

A second major drawback with urinary hydroxyproline measurements that had to be overcome was the fact that this assay measures not only degradation of insoluble tissue collagen but also release from biosynthetic intermediates including degradation of precursors intracellularly. The latter process may account for more than 15 percent of new collagen synthesis (Bienkowski 1984). As the formation of pyridinium crosslinks occurs extracellularly as the final stage of maturation of the collagen fibril, these compounds have unique advantages as markers in reporting the degradation only of mature collagen and not any biosynthetic intermediates.

Table 1. Proportions of free crosslinks in urine from groups of healthy pre- and postmenopausal women and from patients with osteoporosis. Proportion of total, mean *SD*

Group	n	Pyd	Dpd
Measured by HPLC			
Pre-menopausal women	53	38.3 *5.1*	42.4 *6.8*
Post-menopausal women	56	40.1 *4.7*	43.2 *8.0*
Osteoporosis	48	42.6 *5.3*	46.5 *7.0*
Measured by ELISA			
Post-menopausal women	54		44.0 *9.8*

The HPLC values were determined by expressing the free Pyd and Dpd concentrations as a percentage of the total amounts determined after acid hydrolysis. The proportion of free Dpd in a separate group of postmenopausal women was determined similarly except that a specific ELISA for Dpd (Robins et al. 1994a) was used.

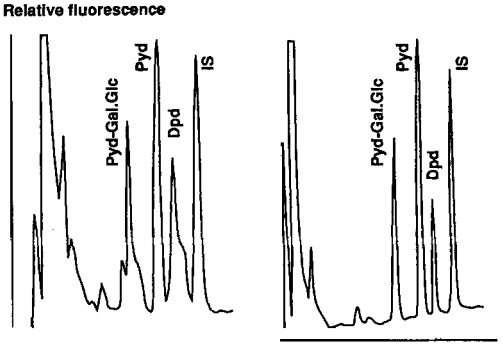

Figure 1. Analysis of free pyridinium crosslinks by HPLC after fractionation on cellulose columns using an automated system (Pratt et al. 1992). Left panel: analysis using the standard conditions developed for urine hydrolysates showing interference by unknown fluorescent components in the area of the crosslinks; right panel: the same urine sample fractionated with additional washing of the cellulose pre-column with mobile phase solvent.

With these advantages in terms of specificity and sensitivity, the measurement of urinary crosslinks has been used widely in recent years. Their application is also facilitated by the lack of any requirements for dietary restrictions (Colwell et al. 1993). It should be borne in mind, however, that it is the use of the crosslinks, and particularly the Dpd crosslink, that confers the increased specificity and that, in a number of recent developments that do not require the presence of the crosslinks, some of the potential advantages may be compromised.

Development of methodology for crosslink measurement

Following the initial identification in urine of collagen crosslinks using either chromatographic separations (Gunja-Smith et al. 1981) or an ELISA method (Robins 1982b), the main interest in the technique came with the development of an HPLC method applicable to measurements of pyridinium crosslinks in urine (Black et al. 1988). Development of an automated HPLC system including an internal standard has improved the precision of the assay by at least 3-fold (Pratt et al. 1992). Initially, measurements of total crosslinks were performed after acid hydrolysis of the urine and these techniques were able to confirm the validity of the crosslinks as bone resorption markers (Robins et al. 1991a). In particular, these assays were shown to correlate closely with bone histomorphometry (Delmas et al. 1991) and with radioisotopic methods (Eastell et al. 1990).

The HPLC assay was used to show that about 40% of the pyridinium crosslinks were present in urine in the free form and could be measured without hydrolysis (Robins et al. 1990, Robins et al. 1991b). The proportion of free crosslinks was found to be relatively consistent for both healthy individuals and for patients groups having osteoporosis, thyroid disorders, hyperparathyroidism and arthrosis. These observations paved the way for the development of direct immunoassays that initially measured both crosslinks and very small (Mr<1000) peptides (Seyedin et al. 1993) and, subsequently, of a monoclonal antibody-based assay for the bone-specific crosslink, Dpd (Robins et al. 1994a).

Proportions of free crosslinks

Some more recent data on the proportions of free crosslinks in different groups are shown in Table 1. For healthy individuals, there was a slightly higher proportion of free crosslink in women than in men (Seibel et al. 1994) but urinary analyses for pre- and post-menopausal women showed no significant difference between these groups, either by HPLC or by ELISA (Table 1). This finding is in contrast to a recent report (Garnero et al. 1995) in which a significantly lower proportion of free Dpd was found in postmenopausal women compared with a premenopausal group and there appeared to be a significant decline in the proportion of free Dpd with increasing output of total Pyd. These results are difficult to reconcile but one possibility is that the measurements of free Dpd have been confounded by interference during the HPLC separation. Analysis of free urine by the standard method used for hydrolysates often led to problems in quantification of the Dpd peak which

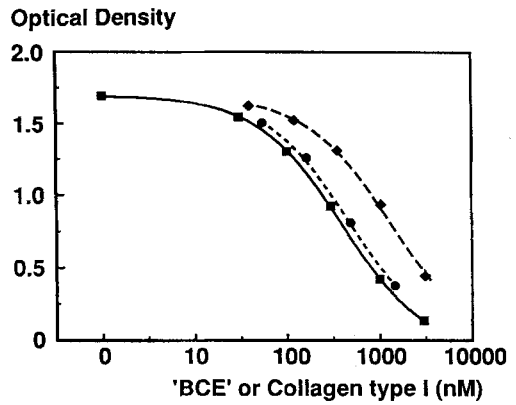

Figure 2. Comparison of the reactivity of collagenase-derived peptides from human bone and skin with an NTx assay (Osteomark™, Ostex International). Both tissues were powdered in a liquid nitrogen mill and were defatted with chloroform:methanol (3:1 v:v); the bone was demineralised with 0.5M-EDTA, pH 8.0 at 4 °C for 5 d. Each tissue was subjected to exhaustive digestion with bacterial collagenase (Type 1A; Sigma) at an enzyme substrate ratio of 1:50 in 0.2M-ammonium bicarbonate – 1μM-$CaCl_2$ at 37 °C for 24h. After centrifugation at 45,000g for 10 min, serial dilutions of the skin (●) and bone (◆) digests were tested in the assay in comparison with the standards provided (■). Aliquots of the supernatant solutions were assayed for hydroxyproline after hydrolysis in 6M-HCl to determine collagen concentrations assuming 300 residues hydroxyproline per molecule collagen.

is relatively small compared with that for Pyd (Figure 1). Any interference of this type which is restricted to measurements of unhydrolysed urine would lead to an overestimation of the proportion of free crosslink in urine. Modification of the sample preparation procedures can often obviate these problems (Figure 1).

Comparison with other assays

More recently, a number of other assays have been developed based not on the crosslinks themselves but on peptides derived from amino acid sequences in the vicinity of the crosslinks. The NTx assay (Hanson et al. 1992) is based on a monoclonal antibody recognising a peptide comprising part of the N-terminal telopeptide. Although the antibody was shown not to react with the linear sequence in the telopeptide, the presence of the pyridinium crosslink was not necessary for reaction with the antibody (Hanson et al. 1992). The presence of sequences from the α2(I)-chain telopeptide in the epitope recognised by the antibody was stated to confer specificity for bone collagen. The assay procedure was standardised in terms of 'Bone Collagen Equivalents' by reaction with peptides derived from a digest of a known quantity of bone (Hanson et al. 1992). In examining the specificity of this assay, however, we found that the reaction with peptides from a digest of human skin with bacterial collagenase was equivalent to that of the bone-derived standard and somewhat stronger than a collagenase digest of EDTA-demineralised bone prepared in the same way as the skin digest (Figure 2). The NTx assay therefore appears to react generally with collagen type I peptides rather than being specific for bone collagen, thus illustrating the importance of using the 3-hydroxypyridinium crosslink, Dpd, to gain specificity for bone.

As an alternative approach assays based largely on the C-terminal telopeptides have been developed. Antibodies to a 12kDa peptide derived from collagenase digestion of bone collagen form the basis of the ICTP assay (Risteli et al. 1993). As a serum assay, this method is probably not directly comparable with the other assays being considered in this section. By raising polyclonal antibodies against a synthetic octapeptide encompassing the (hydroxy)lysine residue at position 17 of the C-terminal telopeptide of collagen type I, Bonde et al. (1994) developed an inhibition ELISA that showed statistically significant correlations with pyridinium crosslink measurements when applied to urine. Again, however, because the presence of bone crosslinks is not essential for reactivity, the specificity of the assay for bone has yet to be demonstrated.

Standardisation

Although few direct comparisons between the different assays are available, some measure in absolute terms of the relative values can be obtained by considering the published normal values for urinary excretion for a relatively homogeneous group, that of adult males. It is useful here to use the concept of Bone Collagen Equivalents (BCE) by converting the measured amount of analyte to its bone collagen equivalent. For the Dpd assay, standardisation is with respect to a compound that has been fully characterised by n.m.r., mass spectrometry and UV spectroscopy. For the study group, the mean excretion corresponds to 140 nmoles BCE per mmole creatinine. The corresponding value for the NTx assay is 40 nmoles BCE per mmole creatinine but this is a somewhat arbitrary figure as the material used for standardisation (collagenase-derived peptides of bone) is not the same as that actually being measured in urine by the assay. The urinary excretion in adult males for the CTx assay, for which the standard is the synthetic octapeptide used as antigen, gives a value of about 100 nmoles BCE per mmole creatinine, but again this figure is unreliable in the absence of further knowledge of the reactive species in urine. There are therefore considerable differences between the various

assays in the apparent amount of bone being resorbed and, although this may not be surprising given the nature of the assumptions made, it does highlight the need for additional comparative studies to evaluate their potential usefulness.

Effects of treatment

A major potential benefit of biochemical markers is the ability to monitor changes in bone resorption following therapy, whether this be with hormones or with disease modifying drugs such as bisphosphonates. In evaluating different biochemical markers, it is important to be sure that the observed changes are reflecting true changes in bone resorption and not simply a metabolic phenomenon that alters the patterns of the analytes being measured. Studies of the effects of estrogen on bone resorption have generally been reflected in differences between pre- and postmenopausal women in urinary pyridinium crosslink concentrations of 50–80 percent (Robins et al. 1994b; Garnero et al. 1994). Somewhat similar values have been obtained for the telopeptide assays (Garnero et al. 1994; Gertz et al. 1994). For patients treated with bisphosphonates, however, the telopeptide assays have shown larger decreases in urinary concentrations (60–80%) compared with the pyridinium crosslinks, for which the decline was about 40 percent (Garnero et al. 1994). More recent data suggest that, in patients with Paget's disease or osteoporosis receiving treatment with bisphosphonates, the percentage decrease in telopeptide assays was 3- to 4-fold higher than that for total pyridinium crosslinks whereas, for these groups of patients, minimal changes in free crosslinks were detected (Garnero et al. 1995).

The effects of treatment may be manifest at several stages in the degradative metabolism of bone collagen (Figure 3). The primary aim in applying biochemical markers is to gain information on the true change in bone resorption. It is generally accepted that measurements of total pyridinium crosslinks provide the closest approach to an absolute measure of bone resorption because the interpretation of these results requires the fewest assumptions. Where changes much greater than those for total pyridinium crosslinks are detected, such as occurs for the telopeptide assays after bisphosphonate treatment, the most likely explanation is that the treatment has also affected a pathway in the degradative metabolism of bone collagen to alter the proportions of small peptides and free crosslinks (Figure 3). This could occur within bone or in the kidney where the brush border was shown in rats to be capable of degrading small peptides (Rucklidge et al. 1988). The proportion of

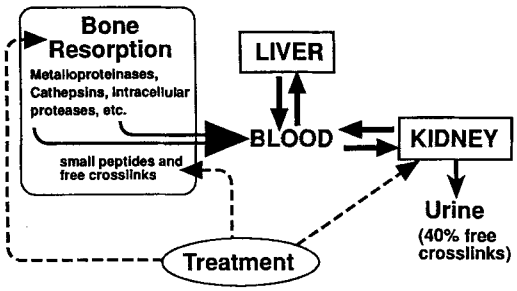

Figure 3. Possible sites of action of the effects of treatment on bone resorption and the degradative metabolism of the cross-linked collagen released.

free crosslinks in blood is similar to that in urine and it is, therefore, unlikely that further degradation to free crosslinks occurs in the kidney. Although changes in the proportions of free crosslinks in urine can be measured, any change in the proportion of reactive peptides in the telopeptide assays relative to total urinary crosslinks is not readily assessed but, as these peptides constitute a relatively small proportion of the total pyridinium crosslink material, it is conceivable that a small change in their relative proportion could result in a large difference in the apparent rate of bone resorption measured by the assay.

In summary, therefore, further knowledge of the degradative metabolism of bone collagen is essential before the information gained from the different biochemical markers for bone resorption can be fully evaluated and interpreted.

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