Biochemical markers of bone turnover

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Invasive techniques measuring bone turnover have provided useful information but all have limitations. Histomorphometry of the iliac crest provides unique information on the rate of formation both at the cell and at the tissue levels, allows to measure the activation frequency of remodeling units but the assessment of bone resorption is less accurate. In addition, measurement of bone turnover is limited to a small area of the cancellous and of the cortico-endosteal envelope which may not always reflect bone turnover of other sites of the skeleton (Delmas 1988). Calcium kinetic studies have allowed quantification of the increase of bone turnover after the menopause but measurement of calcium accretion rate—an index of bone formation- may be inaccurate in elderly women (Eastell et al. 1988). Finally the whole body retention of labeled bisphosphonates (WBR), a marker of bone turnover and bone formation has not proved to be very sensitive (Thomsen et al. 1987). These limitations, in addition to the need of non-invasive techniques that can be applied more widely and repeated several times in a single patient, provide the rationale to develop markers of bone turnover to be measured in blood and urine.

The rate of formation or degradation of the bone matrix can be assessed either by measuring an enzymatic activity of the bone forming or resorbing cells—such as alkaline and acid phosphatase activity—or by measuring bone matrix components released into the circulation during formation or resorption (Table 1). Bone markers cannot discriminate between turnover changes of a specific skeletal envelope, i.e. trabecular versus cortical, but reflect the overall net changes of resorption and formation, which can be seen as an advantage or as a disadvantage. As discussed below, these markers are of unequal specificity and sensitivity, which implies that estimating the net excess of resorption over formation, by comparing the increase of a resorption marker and of a formation marker expressed in percentage or in standard deviation (Z and T scores), can lead to wrong conclusions. Circulating levels of these markers can be influenced by factors other than bone turnover such as their metabolic clearance (liver uptake, renal excretion and/or trapping on bone hydroxyapatite), and by the pattern of immunoreactive moieties recognized by a given antibody. Thus, each of these assays need to be carefully validated in a specific clinical situation before conclusions about their clinical utility can be drawn. Clearly, data obtained in other metabolic bone diseases cannot be readily extrapolated to osteoporosis.

Biochemical markers of bone formation

Serum alkaline phosphatase

The skeletal alkaline phosphatase is an enzyme localized in the membrane of the osteoblasts. It is released into the circulation by an unclear mechanism. Among the several tissues containing alkaline phosphatase, the liver and bone isoenzymes are the major contributors to the serum levels. The intestinal isoenzyme of alkaline phosphatase can also account for part of the circulating levels in some non fasting patients, and the placental alkaline phosphatase circulates during pregnancy. Serum total alkaline phosphatase activity is the most commonly used marker of bone formation but for the above reasons it lacks sensitivity and specificity. Nevertheless, several studies have shown that

Table 1. Biochemical markers of bone turnover

Formation
Serum
Osteocalcin (Bone Gla-Protein)
Total and bone specific alkaline phosphatase
Procollagen I carboxy-terminal extension peptide
Resorption
Plasma
Tartrate-resistant acid phosphatase
Pyridinoline and pyridinoline containing peptides (?)
Urine
Urinary pyridinoline and deoxypyridinoline (collagen cross-links) and containing peptides.
Fasting Urinary calcium and hydroxyproline
Urinary hydroxylysine glycosides

its activity increases with aging in adults, especially in women after menopause (Crilly et al. 1980). In patients with vertebral osteoporosis, values are either normal or slightly elevated and poorly correlated with bone formation determined by iliac crest histomorphometry (Brown et al. 1987, Podenphant et al. 1987). In an attempt to improve the specificity and the sensitivity of serum alkaline phosphatase measurement, techniques have been developed to differentiate the bone and the liver isoenzymes which only differ by posttranslational modifications as they are coded by a single gene. These techniques rely on the use of differentially effective activators and inhibitors, separation by electrophoresis and indirect separation by liver-specific antibodies (Moss 1982, Farley et al. 1981, Duda et al. 1988). In general, these assays have slightly enhanced the sensitivity of this marker, but most of them are indirect and/or technically cumbersome. A real improvement has been obtained recently by using a monoclonal antibody recognizing preferentially the bone isoenzyme (Hill and Wolfert 1986). We have shown that a two site immuno-radiometric assay based on this antibody has a low (16%) crossreactivity with the circulating liver isoenzyme and is highly correlated with the electrophoretic assessment of the bone isoenzyme in patients with Paget's disease (Garnero and Delmas 1993). It is a sensitive marker of the increased bone turnover in postmenopausal women and is an accurate index of the effects of antiresorptive drugs on bone turnover as discussed below.

Serum osteocalcin (BGP)

Osteocalcin, also called bone gla-protein (BGP), is a small non collagenous protein which is specific for bone tissue and dentin, but its precise function remains unknown (Price 1987). Osteocalcin is predominantly synthesized by the osteoblasts and incorporated into the extracellular matrix of bone, but a fraction of neosynthesized osteocalcin is released into the circulation where it can be measured by radioimmunoassay (Price et al. 1980, Price et al. 1981, Lian and Gundberg 1988, Delmas et al. 1983). Circulating osteocalcin has a short half life and is rapidly cleared by the kidney (Price et al. 1981, Delmas et al. 1983). Serum osteocalcin correlates with skeletal growth at the time of puberty, and is increased in a variety of conditions characterized by increased bone turnover such as primary and secondary hyperparathyroidism, hyperthyroidism, Paget's disease and acromegaly. Conversely, it is decreased in hypothyroidism, hypoparathyroidism, and in glucocorticoid-treated patients and in some patients with multiple myeloma and malignant hypercalcemia (reviewed by Delmas 1990). Comparisons of serum osteocalcin with iliac crest histomorphometry and calcium kinetics data have shown that in most conditions, serum osteocalcin is a valid marker of bone turnover when resorption and formation are coupled, and is a specific marker of bone formation whenever formation and resorption are uncoupled (Delmas 1990, Brown et al. 1984, Charles et al. 1985, Delmas et al. 1986).

Because antibodies directed against bovine osteocalcin cross react with human osteocalcin, most systems have been developed with bovine osteocalcin as a tracer, standard and immunogen. Depending on the epitopes recognized by the antibody, some polyclonal antisera—but also monoclonal antibodies—may see, in addition to the intact molecule, fragments of osteocalcin, the significance of which is still unclear (Gundberg and Weinstein 1986, Tracy et al. 1990, Taylor et al. 1990). Using a battery of monoclonal antibodies directed against the various epitopes of the human osteocalcin molecule, we have found that the intact molecule represents about a third of the immunoreactivity in the adult serum (or plasma). A third is represented by several small fragments and another third by a large N terminal-mid molecule fragment (N-mid fragment; Garnero et al. 1994). It is generated in vitro by osteoblastic cells in culture, and circulates in vivo. After few hours at room temperature, a significant fraction of plasma intact osteocalcin is rapidly converted into the large N-mid fragment, resulting in a significant loss of immunoreactivity with most polyclonal antibodies because they recognize the C-terminal end of the molecule. We believe that this pattern could account for some of the discrepancies reported in the literature (Delmas et al. 1990), and for the surprisingly very wide scatter of individual values observed in several studies. From a practical point of view, measuring both the intact molecule and the N-Mid fragment with an assay using appropriate antibodies result in a more robust and sensitive assay.

Procollagen I extension peptides

During the extracellular processing of type I collagen, there is a cleavage of the aminoterminal (p coll-I-N) and carboxyterminal (p coll-I-C) extension peptides prior to the fibril formation. These peptides, also referred as the procollagen carboxy-terminal propeptide (PICP) and amino-terminal propeptide (PINP), circulate in blood where they might represent useful markers of bone formation, as collagen is by far the most abundant organic component of bone matrix. Serum PICP is weakly correlated with histological bone formation, with r values ranging from 0.36 to 0.50, in patients with vertebral osteoporosis (Parfitt et

al. 1987). The menopause induces a significant but marginal (+20%) increase in serum PICP which is not correlated with the subsequent rate of bone loss measured by densitometry (Krane et al. 1977). Contrasting with a significant increase of both serum bone alkaline phosphatase and osteocalcin in patients with vertebral osteoporosis that were treated with fluoride, serum p coll-I-C decreased under therapy (Ebeling et al. 1992). The reasons for this lack of sensitivity are not clear. The metabolism of this peptide in humans is unknown, although it has been shown in the rat that PICP is bound and internalized by the endothelial cells of the liver through the mannose receptor (Smedsrod et al. 1990). Characterization of the circulating immunoreactive forms of PICP may help to clarify these uncertainties.

Biochemical markers of bone resorption

Fasting urinary calcium, hydroxyproline and hydroxylysine glycosides

Fasting urinary calcium measured on a morning sample and corrected by creatinine excretion is certainly the cheapest assay of bone resorption. It is useful to detect a marked increase of bone resorption but lacks sensitivity. Fasting urinary calcium reflects the amount of calcium released during resorption, but also the renal handling of calcium that is influenced by calcium regulating hormones and by estrogens. Hydroxyproline is found mainly in collagens and represents about 13% of the amino acid content of the molecule (Prockop and Kivirikko 1968). Hydroxyproline is derived from proline by a post-translational hydroxylation occurring within the peptide chain. Because free hydroxyproline released during degradation of collagen cannot be reutilized in collagen synthesis, most of the endogenous hydroxyproline present in biologic fluids is derived from the degradation of various forms of collagen (Prockop et al. 1979). As half of human collagen resides in bone, where its turnover is probably faster than in soft tissues, excretion of hydroxyproline in urine is regarded as a marker of bone resorption. Actually, the C1q fraction of the complement contains significant amounts of hydroxyproline and could account for up to 40 % of urinary hydroxyproline (Krane et al. 1977). The relationship of urinary hydroxyproline to the metabolism of collagen is more complex. Hydroxyproline is present in biologic fluids in different forms. About 90% of the hydroxyproline released by the breakdown of collagen in the tissues, and especially during bone resorption, is degraded to the free amino acid that circulates in plasma, is filtered, and is almost entirely reabsorbed by the kidney. It is eventually completely oxidized in the liver and is degraded to carbon dioxide and urea (Kivirikko 1983, Lowry et al. 1985). It has been estimated that the urinary total hydroxyproline may represent only about 10% of total collagen catabolism. Hydroxyproline is present in urine as a free aminoacid and in peptide forms of various molecular weights. Colorimetric assay of hydroxyproline is usually performed on a hydrolyzed urine sample and therefore reflects the total excretion of the aminoacid. As a consequence of its tissue origin and metabolism pattern, urinary hydroxyproline is poorly correlated with bone resorption assessed by calcium kinetics or bone histomorphometry (Delmas 1990). The sensitivity of the assay can be improved by high pressure liquid chromatography (HPLC) but there is an obvious need for a more convenient and sensitive marker of bone resorption.

Hydroxylysine is another amino acid unique to collagen and proteins containing collagen-like sequences. Like hydroxyproline, hydroxylysine is not reutilized for collagen biosynthesis, and although it is much less abundant than hydroxyproline, it is a potential marker of collagen degradation (Krane et al. 1977). Hydroxylysine is present in part as galactosyl-hydroxylysine and in part as glucosyl-galactosyl-hydroxylysine. The relative proportion and total content of galactosyl hydroxylysine and glucosyl-galactosyl hydroxylysine varies in bone and soft tissues which suggests that their urinary excretion might be a more sensitive marker of bone resorption than urinary hydroxyproline. Urinary galactosyl hydroxylysine increases with aging (Moro et al. 1988). This marker deserves further evaluation in osteoporosis but its development is limited by the current HPLC technique.

Plasma tartrate-resistant acid phosphatase

Acid phosphatase is a lysosomal enzyme that is present primarily in bone, prostate, platelets, erythrocytes, and spleen. These different isoenzymes can be separated by electrophoretic methods, which lack sensitivity and specificity. The bone acid phosphatase is resistant to L(+)-tartrate, whereas the prostatic isoenzyme is inhibited (Li et al. 1973). Acid phosphatase circulates in blood and shows higher activity in serum than in plasma because of the release of platelet phosphatase activity during the clotting process. In normal plasma, tartrate-resistant acid phosphatase (TRAP) corresponds to plasma isoenzyme 5, which originates partly from bone, as osteoclasts contain a tartrate-resistant acid phosphatase that is released into the circulation (Minkin 1982). Plasma TRAP is increased in a variety of metabolic bone disorders

with increased bone turnover (Seibel et al. 1992), is elevated after oophorectomy (Stepan et al. 1987) and in vertebral osteoporosis but it is not clear whether this marker is actually more sensitive than urinary hydroxyproline (Stepan et al. 1987). The lack of specificity of plasma TRAP activity for the osteoclast, its unstability in frozen samples, the presence of enzyme inhibitors in serum, are potential drawbacks which will limit the development of enzymatic assays of TRAP in osteoporosis. Conversely, the development of new immunoassay using monoclonal antibodies specifically directed against the bone isoenzyme of TRAP (Kraenzlin et al. 1990) should be valuable to assess the ability of this marker to predict osteoclast activity in osteoporosis.

Urinary excretion of the collagen pyridinium crosslinks and associated peptides.

Pyridinoline (Pyr) and deoxypyridinoline (D-Pyr) also called respectively hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) are the two non reducible pyridinium crosslinks present in the mature form of collagen (Fujimoto et al. 1978). This post-translational covalent cross-linking generated from lysine and hydroxylysine residues is unique to collagen and elastin molecules. It creates interchain bonds which stabilize the molecule within the extracellular matrix. The concentration of Pyr and D-Pyr in connective tissues is very low and varies dramatically with tissue type (Eyre 1987). The highest concentration of Pyr (expressed in mol/mol of collagen) is found in articular cartilage, while D-Pyr is absent from this tissue (Eyre et al. 1984, Eyre et al. 1988). Pyr and D-Pyr are present in tendon and aorta but absent from the skin, an abundant source of type I collagen (Seibel et al. 1992). Because bone is by far the most abundant source of collagen matrix and because its rate of turnover is markedly higher than some other connective tissues such as cartilage, Pyr and D-Pyr concentrations in biological fluids are likely to be predominantly derived from bone. The relative proportion of Pyr and D-Pyr in bone matrix is variable according to the species. Pyr and D-Pyr are released from bone matrix during its degradation by the osteoclasts. As both crosslinks result from a post-translational modification of collagen molecules, they cannot be reutilized during collagen synthesis. Although limited, available data suggest that Pyr and D-Pyr are not metabolized in vivo. They are excreted in urine in free form (about 40%) and in peptide bound form (60%) and the total amount can be measured by fluorimetry after reversed phase HPLC of a cellulose-bound extract of hydrolyzed urine (Eyre et al. 1984, Black et al. 1988).

Urinary Pyr and D-Pyr are markedly higher in children than in adults (Beardsworth et al. 1990), are increased by 50 to 100% at the time of menopause, and go down to premenopausal levels under estrogen therapy (Uebelhart et al. 1991). In patients with vertebral osteoporosis, the urinary crosslink levels, especially of D-Pyr, are correlated with bone turnover measured by calcium kinetics (Eastell et al. 1990) and bone histomorphometry (Delmas et al. 1991), contrasting with poor correlations obtained with urinary hydroxyproline. The urinary excretion of Pyr and D-Pyr is also increased in a variety of metabolic bone diseases with increased bone turnover such as primary hyperparathyroidism, hyperthyroidism and Paget's disease (Uebelhart et al. 1990, Robins et al. 1991). In summary, the measurement of urinary crosslinks appear to have several potential advantages over hydroxyproline: they are relatively specific for bone turnover, they do not appear to be metabolized in vivo prior to their urinary excretion, and the absence of intestinal absorption of Pyr and D-Pyr contained in gelatin allows to collect urine without any food restriction .

The contribution of connective tissues other than bone in the urinary excretion has not been investigated in humans but the fact that for a given assay the molar ratio of Pyr/D-Pyr is similar in normal individuals and in patients with various metabolic bone diseases suggest that Pyr and D-Pyr do not differ markedly in terms of sensitivity. Several studies have shown a circadian rhythm of bone turnover markers characterized by a nocturnal increase—with a peak at 2–5 hr—and a nadir in the early afternoon. Contrasting with a relatively small peak to nadir delta for osteocalcin and PICP, the circadian rhythm of urinary crosslink excretion is quite pronounced, with a mean 30% decrease between 6 hr and noon (Schlemmer et al. 1992, Eastell et al 1992), suggesting that timing of urine collection should be standardized. We have found that measurements of pyridinoline on the first morning void (i.e. urine of the night) and on the second morning void (i.e. "spot" urine sample collected before 6:00 a.m) are highly correlated and not significantly different (unpublished data). The significance of the nocturnal increase of bone turnover is unclear. Patients with vertebral osteoporosis have a greater nocturnal peak with persistence into the following morning collection period than age-matched controls, suggesting a preferential increase of bone resorption at night (Schlemmer et al. 1992).

So far, most of the data have been obtained with the HPLC assay of the total Pyr and D-Pyr excretion. More convenient techniques are required for a broad clinical use of this marker. Immunoassays have been

recently developed, using antibodies directed against either free Pyr and/or D-Pyr (Seyedin et al. 1993, Robins et al. 1994) or against peptides of the cross-linking domains of type I collagen. Assays have been developed with antibodies raised against the N-telopeptide to helix domain (Hanson et al. 1992), the C-telopeptide to helix (Ristelli et al. 1993) and against a linear sequence of the C-telopeptide (Bonde et al. 1994). These immunoassays are based on antibodies recognizing different moieties, the metabolic clearance of which is unknown. They are likely to have different clinical significance and need to be characterized in various metabolic bone diseases. Urinary free Pyr (Pyrilink™) is highly correlated with the total Pyr excretion measured by HPLC, increases significantly after the menopause and in patients with active Paget's disease of bone (Delmas et al. 1993). The ELISA of free D-Pyr using a monoclonal antibody that does not crossreact with Pyr is highly correlated with total D-Pyr measured by HPLC and provides fivefold higher values in children than in normal adults, is increased in osteoporotic patients compared to age-matched controls and in diseases characterized by increased bone turnover such as primary and secondary hyperparathyroidism, Paget's disease and bone metastases from breast cancer (Robins et al. 1994). The three immunoassays developed against crosslinking peptides have been evaluated in several studies. The urinary ELISA developed against the N-telopeptide to helix (Osteomark™) and against the C-telopeptide (CrossLaps™) are markedly increased after the menopause and in patients with hyperthyroidism, primary hyperparathyroidism and Paget's disease (Hanson et al. 1992, Garnero et al. 1994). The serum level of the C-telopeptide to helix but surprisingly not in pagetic patients (Eyres et al. 1992). This marker is increased in osteoporotic patients treated with anabolic steroids that are presumed to decrease bone resorption and to increase collagen synthesis (Hassager et al. 1994). Taken together, these data suggest that serum ICTP is an index of collagen turnover, but not a marker specific of bone resorption. Thus, with one exception, most ELISA appear to be valid alternatives to the conventional cumbersome HPLC assay and their relative sensitivity in osteoporosis is discussed below. The fraction of free and peptide-bound pyridinoline crosslinks excretion, however, does not appear to be constant. We have found that increased bone turnover is associated with an increase of the peptide bound/total fraction and with a decrease of the free/total fraction, although the absolute values of both free crosslinks and peptide-bound crosslinks are increased (Garnero et al. 1994).

Conclusion

The immunoassays of osteocalcin and bone alkaline phosphatase represent so far the most effective markers of bone formation in osteoporosis. The measurement of pyridinium crosslinks, and of some of its related peptides, is the most tangible improvement in the assessment of bone resorption, and well-characterized immunoassays are valuable alternatives to the HPLC technique. Several cross-sectional and longitudinal studies performed in postmenopausal women suggest that increased bone turnover is associated with increased bone loss (Slemenda et al. 1987, Johansen et al. 1988). The prediction of fractures with bone turnover markers is a challenge because it requires long term studies in large populations, but the few available data are consistent and encouraging (Hansen et al. 1991, Szulc et al. 1993). Bone markers are likely to improve the efficacy of assessing the risk of osteoporosis by bone mass measurement, but those postmenopausal women that should be targeted for such a combined approach need to be defined. Because of the relatively small change of bone mass in patients treated with antiresorptive therapy, monitoring of treatment efficacy by DXA in the individual patient is a challenge and repeated bone marker measurement during treatment is likely to improve the management of such patients (Garnero et al. 1994).

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