NEFROLOGIA. Vol. XV. Suplemento 1, 1995

The assessment of bone metabolism in vivo using biochemical approaches

R. G. G. Russell
Department of Human Metabolism & Clinical Biochemistry, University of Sheffield Medical School, Beech Hill Rd., Sheffield, S10 2RX. UK.

Introduction

The monitoring of organ function by the measurement of tissue-specific biochemical products in body fluids is a well established principle in clinical biochemistry. In the case of bone, such measurements have been routinely limited to only a few assays, notably alkaline phosphatase to provide an indirect measure of osteoblast activity. However, many other products derived from the increased metabolism of bone appear in serum or urine where they can be measured as indicators of bone turnover and disease activity.

There have been a number of significant developments in this area in recent years. This is linked to the increasing interest in metabolic bone diseases particularly osteoporosis, in which the use of biochemical assays for the diagnosis and monitoring of treatment are needed. This brief review will provide an outline of the current status and some anticipated developments in this area.

Desirable features of a tissue marker

Biochemical markers of bone turnover measured in plasma or urine are proteins or products derived from them. In general they are either enzymes derived from osteoblasts involved in bone formation, or from osteoclasts involved in bone resorption, or are constituents of the bone matrix, which escape into the circulation during the process of bone formation, or which are released as breakdown products during resorption (Figure 1).

In assessing the value of any marker, several points need to be kept in mind. Firstly, an ideal marker should be specific to the tissue being monitored. In the case of bone this applies to osteocalcin, but not, for example, to hydroxyproline or its peptides. An ideal marker should also be easily measurable by specific, sensitive and precise techniques in either serum or urine. Other criteria that determine the value of any biochemical marker of tissue metabolism include knowledge of the factors that control its synthesis and metabolism, and its entry into and removal from the circulation. With most markers there is still only limited information available about their metabolism and kinetics and the factors which influence their production and degradation.

Markers of bone formation

The three markers of bone formation in current use are alkaline phosphatase, osteocalcin or bone gla protein, and propeptides derived from type I collagen.

Alkaline phosphatase.

Alkaline phosphatase in serum has been used for more than 50 years to monitor bone metabolism, and is still the most frequently used marker.

Alkaline phosphatase is an ectoenzyme anchored to the cell surfaces of osteoblasts and other cells. The enzyme may be shed initially attached to plasma membrane components. The processes regulating release of the enzyme from cells are poorly understood, but plasma membrane vesicles containing alkaline

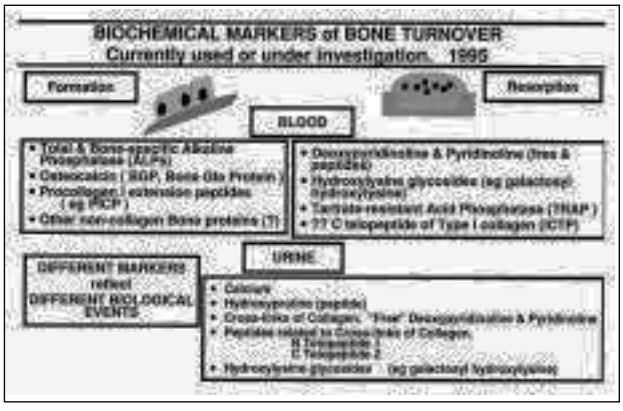

Fig. 1.–Scheme to show the major biochemical markers of bone metabolism.

phosphatase are probably involved in the initiation of mineralisation in skeletal tissues. The clearance from the circulation is relatively slow, with a half-life in the order of 1-3 days for the bone isoenzyme.

Studies of hypophosphatasia, an inherited disease characterised by a deficiency of alkaline phosphatase coupled with defective skeletal mineralisation, gives important clues about the biological function of alkaline phosphatase. In this condition the precise genetic defect is most commonly a point mutation that leads to synthesis of a defective enzyme lacking full catalytic activity. There is an accumulation of several phosphorylated metabolites, notably pyridoxal phosphate, phosphoethanolamine and inorganic pyrophosphate, which therefore are probably among the natural substrates for alkaline phosphatase. The accumulation of inorganic pyrophosphate, an inhibitor of crystal growth of hydroxyapatite, may be directly responsible for the defect in skeletal mineralisation.

The values for alkaline phosphatase in plasma and serum are raised in conditions such as Paget's disease, osteomalacia, and after fractures or ectopic bone formation. However, alkaline phosphatase is not specific to bone, and ideally selective measurement of the bone isoenzyme should be used as a marker of bone formation. In clinical practise the major problem for diagnostic purposes is to distinguish between the isoenzymes derived from liver and bone, although the intestinal enzyme may be raised after meals, and the placental isoenzyme during pregnancy.

Only a single gene encodes for the isoenzymes of alkaline phosphatase found in bone, liver and kidney. Fortunately, however, there are different post-translational modifications made to the enzyme from different tissues after synthesis, resulting in glycosylated products. Thus the bone isoenzyme can be distinguished based on sialic acid residues. The bone isoenzyme can therefore be separated and selectively measured by methods based on differential heat denaturation, electrophoresis, precipitation with wheat germ lectin, and immunoassays with monoclonal or polyclonal antibodies. In this way, specific assays for the bone isoenzyme of alkaline phosphatase are being introduced. Among the best of these are immunoassays, utilising two antibodies, one for capture of the protein and one for its assay. As these specific methods for the bone isoenzyme are becoming more readily available, they are likely to be used more frequently, particularly for distiguishing liver from bone disease, and for population studies or monitoring individuals with bone disease.

In normal individuals about half of the total alkaline phosphatase is derived from bone and the rest from liver. In conditions such as Paget's disease the changes in alkaline phosphatase are often very substantial, so that the dominant circulating form of the enzyme is from bone, meaning that assays of the total enzyme are often sufficient and there is no great advantage in using bone-specific assays. The tissue specific assays come to be much more important in less severely affected patients or in other milder disorders of bone turnover, including osteoporosis, where changes occur predominantly within the normal range.

Values for total alkaline phosphatase show a log-normal distribution. The analytical precision of routine assays for alkaline phosphatase are high, in the order of 1-2 %, and the day to day and month to month biological variation is small. Changes of 10 % or more therefore usually represent significant changes in an individual. However for clinical purposes and in trials of therapies, eg in Paget's disease, a fall of 25 % or more is usually required to indicate a significant clinical response.

Osteocalcin

Osteocalcin, also known as bone Gla protein (BGP), is a bone specific protein, which has proven to be a sensitive and specific marker of osteoblast activity in a variety of metabolic bone diseases (Figure 2). Factors regulating its production from osteoblasts are known in some detail. Its synthesis is dependent upon the presence of active metabolites of vitamin D, especially 1,25-dihydroxyvitamin D and its requires vitamin K for the conversion by carboxylation of three glutamate residues to gammacarboxyglutamate (Gla). These post-translational modifications are similar to those seen in the vitamin K-dependent blood clotting proteins, eg, prothrombin, and confer calcium binding properties on osteocalcin. This can be used to differentiate fully carboxylated from partially carboxylated osteocalcin in the circulation and it has

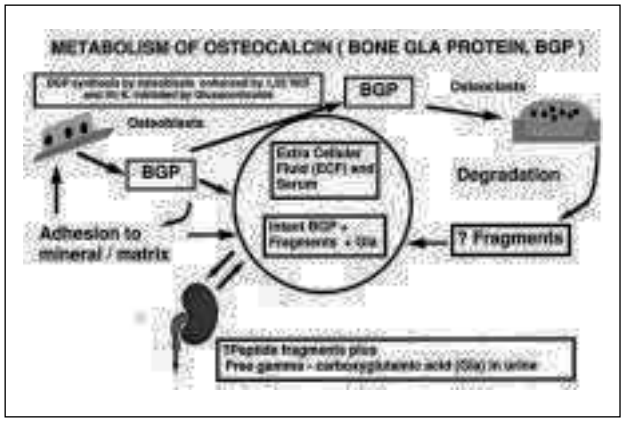

Fig. 2.–Scheme to show the synthesis and metabolism of osteocalcin.

been shown recently that a significant proportion of osteocalcin in osteoporotic elderly patients is incompletely carboxylated. Indeed, the presence of un-decarboxylated osteocalcin is associated with a significantly increased risk of hip fracture in the elderly.

The factors determining the deposition of osteocalcin in bone matrix and its liberation into the circulation are unclear and significant metabolism to peptides and even to free gamma carboxyglutamate (free Gla) occurs. Measurements of the free Gla in plasma and urine have been used in attempts to monitor bone resorption, but appear not to be sufficiently specific or informative.

Measurements of serum osteocalcin by immunoassays show increases in conditions associated with increased bone formation, eg, hyperparathyroidism, hyperthyroidism, bone metastases. In Paget's disease, however, the rises are less than expected, perhaps reflecting differential incorporation into bone matrix, or altered synthesis by osteoblasts. Reduced levels of osteocalcin may reflect lower rates of bone formation, as seen, for example, in myeloma. Osteocalcin values may be substantially reduced during treatment with glucocorticosteroids, although in this case it should be remembered that glucocorticoids specifically suppress osteocalcin synthesis by osteoblasts, while not necessarily similarly depressing collagen synthesis or production of alkaline phosphatase to an equivalent degree.

Serum osteocalcin values can reflect the age-related increase in bone turnover, and the values rise after the menopause and fall after treatment with oestrogens, and also with salmon calcitonin. Such measurements are, therefore, useful in relation to osteoporosis, although much data is based on population studies, rather than changes in individual patients. There are marked differences in the performance of different assays used in different laboratories, some of which are available commercially. New developments include the development of assays utilising antibodies against human rather than bovine osteocalcin, and the use of sandwich assays with two monoclonal antibodies, so that only the intact molecule is measured, rather than fragments. There is also interest in developing non-isotopic methods, and in assays for fragments of osteocalcin that might be released during the resorption of bone matrix.

Procollagen peptides

Collagen is the major structural protein of bone, and comprises about 90 % of the organic material. Collagen clearly contributes to the integrity and strength of bone matrix and defects in its production, eg. in osteogenesis imperfecta, lead to bone of poor quality, susceptible to fracture. Attempts to measure collagen synthesis is, therefore, a more logical approach than measuring other less abundant matrix constituents in the assessment of bone formation. During collagen synthesis pro-peptides are released both from the amino- terminal ('N -terminal') and carboxy-terminal ('C-terminal') ends of the pro-collagen molecule, after the three individual alpha chains have formed the triple helix, which will become part of the collagen fibril (Figure 3).

Assays for both the N- and C- terminal pro-peptides exist. The C-terminal peptide has the advantage of not being significantly retained in bone, unlike the N-terminal peptide. There is therefore considerable interest in the use of assays for the collagen type I C-terminal peptide (PICP) to monitor collagen synthesis related to bone formation. The values are increased during growth and in situations of increased bone formation, such as occur in Paget's disease, and in response to growth hormone. However, the values vary within a relatively narrow range and measurements may be of more use in population studies than in monitoring individual patients. Moreover, propeptides from type I collagen are also derived from skin and other tissues, so it is desirable to develop bone specific assays if this is feasible.

Measurements of bone resorption

Biochemical markers used to monitor bone resorption include urinary measurements of hydroxyproline-containing peptides, hydroxylysine glycosides and pyridinoline crosslinks, that are all derived from collagen. In some circumstances, fasting urine calcium can give an indirect measure of bone resorption rates and may be useful in Paget's disease and in patients

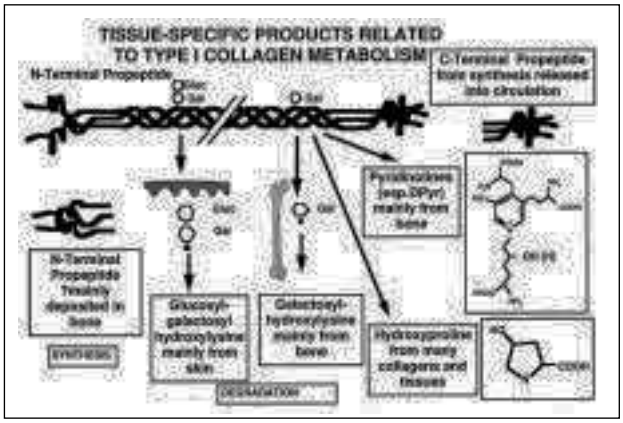

Fig. 3.–Scheme to show the major biochemical markers related to the metabolism of Type I collagen.

with metastatic bone disease for following responses to treatment. Other measurements include assays of tartrate-resistant acid phosphatase, and free gamma carboxy glutamic acid (see above).

Hydroxyproline

Until a few years ago, the best established and most widely used marker of bone resorption was the measurement of hydroxyproline in urine. Peptides containing hydroxyproline are released into urine from the proteolytic breakdown of collagen in bone and other tissues. Since hydroxyproline is one of the abundant amino acids in collagen, its measurement is logical, but hydroxyproline is also found in other proteins and is not specific for collagen in bone. Furthermore, significant amounts of hydroxyproline can be derived from dietary sources of collagen (gelatin), and there is extensive metabolism within the body. For these reasons it is likely that hydroxyproline assays will be eventually replaced by more specific measurements of bone resorption, notably by the use of pyridinoline crosslinks, which do not suffer from these disadvantages.

Nevertheless, measurements of hydroxyproline, particularly in early morning fasting urines, have proved to be useful in evaluating responses in trials of new therapies. Their use in individual patients is handicapped by the low precision/reproducibility of assays, with coefficients of variation (CVs) typically ranging from 10-25 %. A change of 50 % or so may therefore be required in two consecutive measurements before a significant response can be claimed. In Paget's disease the changes that occur are large and there may be little advantage in using pyridinolines instead of hydroxyproline for practical purposes. Several studies in relation to osteoporosis show that, when measurements are made carefully, urinary hydroxyproline values rise after the menopause and fall again when antiresorptive drugs such as oestrogens, calcitonins and bisphosphonates are given.

Pyridinoline crosslinks

Pyridinoline (Pyr) and deoxypyridinoline (DPyr), also called hydroxylysyl pyridinoline (HL) and lysyl pyridinoline (LP) respectively, are currently receiving considerable attention as the most promising markers of bone resorption (Figure 4). Both are non-reducible crosslinks which stabilise the collagen chains within the extracellular matrix and are formed by the condensation of three lysine and/or hydroxylysine residues in adjacent alpha chains. Both Pyr and DPyr are

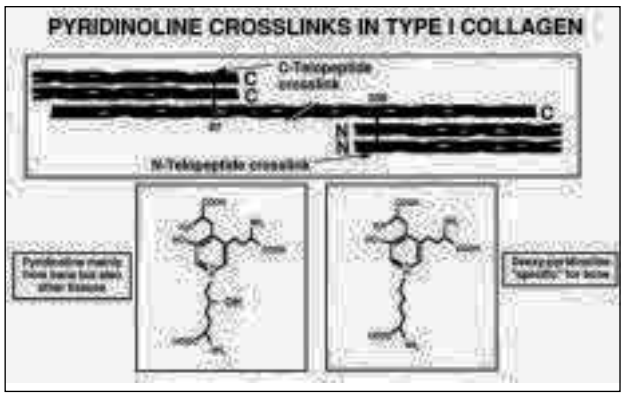

Fig 4.–Scheme to show the position of pyridinoline crosslinks in Type I collagen.

present in bone, but DPyr appears to be found in significant quantities only in bone collagen, making it a potentially specific and more robust marker for bone resorption.

About 40 % of these crosslinks appear free in urine and the remainder are in peptide form. The free or total amount after acid hydrolysis are usually measured by reverse phase HPLC analysis with detection based on the intrinsic fluorescence of these compounds. Although these assays remain the reference methods, these assays are cumbersome and labour-intensive. Fortunately, there has been recent progress in developing immunoassays against the free amino acids or against peptides containing the crosslinks, and these offer considerable hope for producing more rapid and specific assays. At present at least three groups of immunoassays have been reported, one for pyridinolines present as free aminoacids or in small peptides, another for the crosslinked N-telopeptide in urine, and the third for the C-terminal telopeptide in serum. The CVs for these assays are in the order of 5-15 % and there appears to be significant circadian and other sources of biological variation so that further validation is needed.

The pyridinolines do not appear to be significantly absorbed from the diet. It is thought that they are not metabolised, although this remains to be proven. Excretion in the urine may give a quantitative measure of bone resorption since mature collagen contains about 0.07 mols per mole of collagen. However, more information is needed to determine whether there are differences in pyridinoline crosslink contents of collagen in different parts of the skeleton, since it is clear that there are differences at least between species.

As would be expected from a good marker of bone resorption, the values for pyridinolines in urine are increased in childhood and after the menopause. They are also increased in other conditions, eg. in

endocrine (eg. hyperthyroidism) and neoplastic disorders. There are excellent correlations between excretion of crosslinks and bone turnover measured by radioisotope kinetics or bone histomorphometry. There are clear circadian patterns of excretion in these crosslinks with fasting values being in the order of 40 % higher than 24 hour values, which may reflect increases in bone resorption overnight. These circadian changes are of considerable biological interest but pose problems for choosing appropriate urines for assay. At present the recommendation should be to use 24 hour urines whenever possible, although the use of fasting urine assays (ie. collections made over defined 2-3 hour periods in the morning after the overnight urine is voided) may still be informative if taken under strictly controlled conditions.

In the near future, the current development of fast and reproducible immunoassays, either of free pyridinolines or of degradation peptides containing them, are likely to make measurements of pyridinolines as readily available as alkaline phosphatase. Because of their theoretical advantages, they are therefore likely to become established as the measurements of choice for following changes in bone resorption, particularly when the assays are adapted for use in serum rather than urine.

Hydroxylysine glycosides

Hydroxylysine glycosides are also derived from Type I collagens. Hydroxylysine, like hydroxyproline derived from proline, is produced by a post-translational hydroxylation. The subsequent glycosylation of hydroxylysine differs in collagens in different tissues. The monoglycosylated galactosyl hydroxylysine is enriched in bone compared with the diglycosylated form, glucosyl galactosyl hydroxylysine, which is the major form in skin.

Assay is by HPLC, and is therefore technically demanding and relatively slow. Reported measurements, however, suggest that this may be a useful marker of bone resorption, for example, in osteoporosis. Some concern exists over whether the glycosylated forms remain intact during passage down the renal tubule. If specific immunoassays could be developed, hydroxylysine glycosides might offer a useful and practical additional measurement of bone resorption.

Acid phosphatase

Acid phosphatase is a lysosomal enzyme which exists in several forms in different tissues. The type 5 isoenzyme is the one found in osteoclasts, which appear to be released during bone resorption. Assays of total tartrate-resistant acid phosphatase (TRAP, Tartrate-Resistant Acid Phosphatase) in the circulation are moderately raised in disorders associated with increased bone resorption, but the assays are difficult to perform because of the instability of the enzyme and the relatively small changes observed in pathological states. The development of immunoassays specific for the type 5 form offer the potential of producing better assays for monitoring bone resorption.

Other Assays

A variety of other assays in the past, or of current interest, for measuring bone formation and resorption have been tried.

One interesting observation is that serum proteins such as alpha$_2$ HS-glycoprotein, derived from the liver, are deposited in bone and the values fall when bone formation rates are high. However, although this is of physiological interest, the changes are relatively small, and such measurements do not at present provide a practical method of monitoring bone formation rates in individuals.

As more has become known of specific proteins in bone matrix, other assays have been developed and evaluated, eg. for osteonectin and osteopontin, and others may be developed in the future, eg. for other bone sialoproteins, and matrix Gla protein. There is also much interest in the measurement of the many cytokines and growth factors now known to influence bone metabolism (eg. interleukins 1 & 6, tumour necrosis factors [TNFs], insulin-like growth factors I & II and their binding proteins), and in some cases for their soluble antagonists (eg. the IL-1-receptor antagonist) or receptors (eg. the soluble p55 and p75 TNF receptors).

With regard to the current staus of work in this area, there is clearly considerable potential for improving existing assays and developing additional specific assays but more work is needed.

General and specific uses of assays

In the past the major uses for these assays have been to help with the diagnosis and management of patients with florid disorders of bone metabolism, such as Paget's disease, or vitamin D-deficient osteomalacia. However, there is a need to use such assays to monitor the more subtle changes that occur in conditions such as osteoporosis, where the changes in an individual may occur within the normal reference range.

Paget's disease

Paget's disease is the best example of a disease in which biochemical markers of bone metabolism have been extensively used in clinical practise.

In Paget's disease, there have been three major uses for measurements of bone markers:

1) to assess and to monitor disease activity in individual patients;

2) to evaluate dose-response relationships to existing and new drugs in therapeutic trials;

3) to evaluate the value of novel biochemical markers of bone metabolism, compared with established markers.

The major markers in routine use have been alkaline phosphatase and urinary hydroxyproline. Alkaline phosphatase is of proven value in monitoring disease activity in individuals with Paget's disease and is the standard measurement used in clinical trials and for comparison with new markers.

Many other products of bone have been shown to be raised in serum and/or urine in Paget's disease and to be reduced on treatment. These include products associated with bone formation, eg. osteocalcin, osteonectin, N- and C-terminal pro-peptides of Type I collagen, or with bone resorption eg. acid phosphatase, free gamma-carboxy-glutamate, and hydroxylysine glycosides, especially galactosyl-hydroxylysine that appears to be relatively specific for bone. The measurement of these other products of bone metabolism, eg. osteocalcin, and other collagen-derived peptides and bone matrix proteins often show unexpected deviations from the predicted increases, compared with alkaline phosphatase and hydroxyproline, which may reflect aberrant production and metabolism of these products in unkeated Paget's disease. This is an important reminder that different biochemical markers reflect different biological processes. Serum proteins, eg. alpha-2 HS glycoprotein, that have a high affinity for bone may be reduced in the serum of patients with active Paget's disease.

Studies with biochemical markers in Paget's disease have illustrated another very important principle, in that it is possible to demonstrate the dissociation between effects of drugs on bone formation and bone resorption. The major drugs used in Paget's disease are the calcitonins and bisphosphonates, which are inhibitors of bone resorption. It is therefore not surprising that the earliest biochemical changes that occur are in the resorption markers, which can show full responses within only a few days of starting treatment with potent bisphosphonates such as pamidronate. It is very important to realise that, in the routine management of patients, alkaline phosphatase measurements change more slowly in response to treatment than do markers of bone resorption, and that the full response may not be seen for several weeks or even months. Since the currently used potent treatments for Paget's disease, especially with the bisphosphonates, can induce complete biochemical remissions, it is important to realise that judging responses on early changes in alkaline phosphatase alone may give a misleading picture of how effective treatment has been. For this reason, it is wise to advocate that measurements of both alkaline phosphatase and of appropriate bone resorption markers should become routine in the therapeutic management of Paget's disease to ensure adequate responses to treatment.

Osteoporosis. Perhaps the greatest challenge for the future use of markers is in osteoporosis. Here there are two major needs:

1) to identify the patients at greatest risk, eg, those with rapid rates of bone loss compared with bone formation, and

2) to monitor the effects of specific treatment (eg, with oestrogens, calcitonins and bisphosphonates, or with bone forming agents) in individual patients.

Most data related to age, or postmenopausal changes in bone metabolism, are based on populations. Important work from Christiansen's group has shown that a combination of biochemical assays, including total alkaline phosphatase and osteocalcin in plasma, and hydroxyproline and calcium in fasting urine, have strong predictive power in relation to rates of bone loss subsequently measured by bone densitometry techniques. It is of interest that individual biochemical measurements may eadh add to the power of this prediction. This reminds us that each marker reflects different biochemical and physiological processes and may not, therefore, always show identical changes. More work is needed from other laboratories to evaluate the value of such measurements in the assessment of individual patients.

The use of bone markers to monitor individual patients requires assays of high precision. In the case of bone specific alkaline phosphatase, where the precision is in the order of a few percent, this requirement may be easier to meet than with urinary assays for hydroxyproline or pyridinolines where the coefficient of variation for the assays may be in the order of 15 % or so, requiring a treatment-induced change in the region of 30 % or more before a true response can be claimed. The increasing interest in the prevention and management of osteoporosis make the need for improved methods for measurement of these markers a high priority.

Prospects for the future

In addition to the development of novel assays of greater specificity and sensitivity, particularly using monoclonal antibodies, there is a need for more rapid assays for practical clinical use. In view of the socioeconomic importance of bone disease, there is likely to be a considerable investment in this area and the eventual development of fast assays may come.

Although there is a major interest in markers of bone disease, there is also considerable interest in extending these possibilities to other connective tissues, notably cartilage and other joint tissues in relation to destructive arthritis. As with bone, several cartilage specific components exist (eg. COMP, cartilage oligomeric protein) and there is a prospect of measuring cartilage-specific crosslinked collagen peptides, eg. between type IX and type II collagen, which contain pyridinoline crosslinks, to monitor cartilage collagen degradation in articular cartilage. Such assays are in their infancy but offer considerable hope for better diagnosis and evaluation of bone and joint disorders.

Conclusions

The processes of bone formation and resorption can be monitored in vivo by measuring enzymes and other protein products released by osteoblasts and osteoclasts respectively. The major validated biochemical markers of bone formation currently in use include the bone isoenzyme of alkaline phosphatase, osteocalcin (also known as BGP, bone Gla protein) and propeptides derived from the N or C terminal ends of the Type I procollagen molecule. Markers of bone resorption include tartrate resistant acid phosphatase and, in particular, breakdown products of collagen. The longest established of these is the measurement in urine of hydroxproline in collagen peptides, but the assays are cumbersome. Furthermore, hydroxyproline is not specific to bone collagen and is also derived from the diet. There is much current interest in collagen products that are more specific to bone, including galactosyl hydroxylysine, and the collagen crosslinks, pyridinoline and deoxypyridinoline. The pyridinolines appear to be the most promising markers of resorption and may enable quantitative evaluation of rates of bone resorption in man.

These biochemical methods are of use in the diagnosis and evaluation of bone diseases, in population studies, and for monitoring responses to hormones and drugs. It is important to remember that individual markers reflect different biochemical and physiological processes and may not, therefore, always show identical changes.

There is an increasing amount of work being devoted to the study of bone biomarkers, partly because of the current interest in osteoporosis. There are exciting prospects ahead for improvements in technical methods and for the use of new markers derived from bone cells and bone matrix.

Bibliografía

1. Azria M and Russell RGG: Biochemical approaches to the measurement of bone metabolism in vivo. Current Opinion in Orthopaedics, Metabolic bone disease (Ed. M. Andersen, Publ. *Current Science.* 3 No. 1, 103-109, 1992.
2. Black D, Marabani M, Sturrock RD and Robins SP: Urinary excretion of the hydroxypyridinium crosslinks of collagen in patients with rheumatoid arthritis. *Ann Rheum Dis* 48:641-644, 1989.
3. Brixen K, Nielsen HK, Eriksen EF, Charles P and Mosekilde L: Efficacy of wheat germ lectin-precipitated alkaline phosphatase in serum as an estimator of bone mineralization rate: comparison to serum total alkaline phosphatase in serum bone Gla-protein. *Calcif Tissue Int* 44:93-98, 1989.
4. Charles P, Mosekilde L, Risteli L, Risteli J and Eriksen EF: Assessment of bone remodeling using biochemical indicators of type I collagen synthesis and degradation: relation to calcium kinetics. *Bone Miner* 24:81-94, 1994.
5. Colwell A, Russell RGG and Eastell R: Factors affecting the assay of urinary 3-hydroxy pyridinium crosslinks of collagen as markers of bone resorption. *Eur J Clin Invest* 23:341-349, 1993.
6. Delmas PD, Christiansen C, Mann KG and Price PA: Bone Gla protein (osteocalcin) assay standardisation report. *J Bone Min Res* 5:5-11, 1990.
7. Delmas PD, Schlemmer A, Gineyts E, Riis B and Christiansen C: Urinary excretion of pyridinoline crosslinks correlates with bone turnover measured on lilac crest biopsy in patients with vertebral osteoporosis. *J Bone Min Res* 6:639-644, 1991.
8. Delmas PD: Clinical uses of biochemical markers of bone remodeling in osteoporosis. *Bone* 13:S17-S21, 1992.
9. Eastell R, Hampton L, Colwell A, Green JR, Assiri AMA, Hesp R, Russell RGG and Reeve J: Urinary collagen crosslinks are highly correlated with radioisotopic measurements of bone resorption. In: Christiansen C, Overgaard K, eds. *Osteoporosis 1990.* Copenhagen Osteopress: 469- 470, 1990.
10. Eastell R, Robins SP, Colwell T, Assir AMA, Riggs BL and Russell RGG: Evaluation of bone turnover in type I osteporosis using biochemical markers specific for both bone formation and bone resorption. *Osteoporosis Int* 3:255-260, 1993.
11. Eastell RD, Calvo MS, Burrit MF, Offord KP, Russell RGG and Riggs BL: Abnormalities in circadian patterns of bone resorption and renal calcium conservation in type I osteoporosis. *J Clin Endocrinol Metab* 74:487-494, 1992a.
12. Editorial: Pyridinium crosslinks as markers of bone resorption. *The Lancet* 2, 278, 1992.
13. Elomaa I, Virkkunen P, Risteli L and Risteli J: Serum concentration of the cross-linked carboxyterminal telopeptide of type I collagen (ICTP) is a useful prognostic indicator in multiple myeloma. *Br J Cancer* 66:337-341, 1992.
14. Eyre DR: Collagen crosslinking amino-acids. In: *Methods in Enzymology* 144:115-139, 1987.
15. Garnero P and Delmas PD: Assessment of the serum levels of bone alkaline phosphatase with a new immunoradiometric assay in the patients with metabolic bone disease. *J Clin Endocrinol Metab* 77, 1993.

16. Garnero P, Grimaux M, Seguin P and Delmas PD: Characterisation of immunoreactive forms of human osteocalcin generated in vivo and in vitro. *J Bone Min Res* 9:255-264, 1994.
17. Gertz BJ, Shao P, Hanson DA, Quan H, Harris ST, Genant HK, Chesnut CH and Eyre DR: Monitoring bone resorption in early postmenopausal women by an immunoassay for cross-linked collagen peptides in urine. *J Bone Min Res* 9:135-142, 1994.
18. Hanson D, Weis ME, Bollen A-M, Maslan SL, Singer FR and Eyre DR: A specific immunoassay for monitoring human bone resorption: quantitation of type I collagen cross-linked N-telopeptides in urine. *J Bone Min Res* 7:1251-1258, 1992.
19. Hill GS and Wolfert RL: The preparation of monoclonal antibodies which react preferentially with human bone alkaline phosphatase and with lover alkaline phosphatase. *Clin Chem Acta* 186:315-320, 1989.
20. Kraenzlin ME, Lau K-HW, Liang L, Freeman TK, Singer FR, Stepan J and Baylink DJ: Development of an immunoassay for human serum osteoclastic tartrate-resistant acid phosphatase. *J Clin Endocrinol Metab* 71:442-451, 1990.
21. Krane SM, Kantrowitz FG, Byrne M, Pinnell SR and Singer FR: Urinary excretion of hydroxylysine and its glycosides as an index of collagen degradation. *J Clin Invest* 59:819-827, 1977.
22. Masters PW, Jones RG, Purves DA, Cooper EH and Cooney JM: Commercial assays for serum osetocalcin give clinically discordant results. *Clin Chem* 40:358-363, 1994.
23. Moro L, Gazzarrini C, Modricky C, Rovis L, De Bernard B, Galligioni E, Crivellari D, Morassut S and Monfardini S: High predictivity of galactosyl-hydroxylysine in urine as an indicator of bone metastases from breast cancer. *Clin Chem* 36:772-774, 1990.
24. Parfitt AM, Simon LS, Villanueva AR and Krane SM: Procollagen type I carboxy-terminal extension peptide in serum as a marker of collagen biosynthesis in bone. Correlation with iliac bone formation rates and comparison with total alkaline phosphatase. *J Bone Min Res* 2:427-436, 1987.
25. Pratt DA, Daniloff Y, Duncan and Robins SP: Automated analysis of the pyridinium crosslinks of collagen in tissue and urine using solid-phase extraction and reversed-phase high performance liquid chromatography. *Anal Biochem* 207:168-175, 1992.
26. Risteli J, Elomaa I, Niemi S, Novamo A and Risteli L: Radioimmunoassay for the pyridinoline cross-linked carboxy-terminal telopetide of type I collagen: a new serum marker of the bone collagen degradation. *Clin Chem* 39:635-640, 1993.
27. Robins SP, Black D, Paterson CR, Reid DM, Duncan A and Siebel MJ: Evaluation of urinary hydroxypyridinium crosslink measurements as resorption markers in metabolic bone diseases. *Eur J Clin Invest* 21:310-315, 1991.
28. Robins SP: Crosslinking of collagen: isolation, structural characterisation and glycosylation of pyridinoline. *Biochem J* 215:167-173, 1983.
29. Seibel MJ, Duncan A and Robins SP: Urinary hydroxy pyridinium crosslinks provide indices of cartilage and bone involvement in arthritic disease. *J Rheum* 16:964-970, 1989.
30. Szulc P, Chapuy M-C, Meunier PJ and Delmas PD: Serum undercarboxylated osteocalcin is a marker of the risk of hip fracture in elderly women. *J Clin Invest* 91:1769-1774, 1993.
31. Uebelhart D, Gineyts E, Chapuy M-C and Delmas PD: Urinary excretion of pyridinium crosslinks: a new marker of bone resorption in metabolic bone disease. *Bone Mineral* 8:87-96, 1990.
32. Uebelhart D, Schlemmer A, Johansen JS, Gineyts E, Christiansen C and Delmas PD: Effect of menopause and hormone replacement therapy on the urinary excretion of pyridinium crosslinks. *J Clin Endoc Metab* 72:367-373, 1991.
33. Uebelhart D, Gineyts E, Chapuy M-C and Delmas PD: Urinary excretion of pyridinium crosslinks: a new marker of bone disease. *Bone & Mineral* 8:87-96, 1989.