ELSEVIER

Journal of Chromatography B, 781 (2002) 345–358

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Review

Acid phosphatases as markers of bone metabolism

Yoshihiko Igarashi[a,*], Minako Y. Lee[b], Shigeru Matsuzaki[a]

[a]*Dokkyo University School of Medicine, Department of Biochemistry, 321-0923 Mibu Tochigi, Japan*
[b]*University of Washington School of Medicine, Department of Biological Structure, Washington, USA*

Abstract

Various biochemical markers have been used to assess bone metabolism and to monitor the effects of treatments. Tartrate resistant acid phosphatase (TRAP; EC 3.1.3.2) has often been used to assess bone absorption. Although osteoclasts contain abundant TRAP and they are responsible for bone resorption, the total TRAP activities in the serum measured by colorimetric methods little reflect the bone turnover. TRAP 5 is further separated into 5a and 5b by electrophoresis. Type 5b is considered to be derived from the osteoclast, and therefore attempts are being made to measure exclusively serum TRAP 5b by kinetic methods, immunological methods, and chromatographic methods including ion-exchange and heparin column chromatography.

Keywords: Reviews; Bone metabolism; Acid phosphatases

Contents

*Corresponding author. Tel.: +81-28-287-2127; fax: +81-28-286-7268.
E-mail address: bioiga@dokkyomed.ac.jp (Y. Igarashi).

1570-0232/02/$ – see front matter
PII: S1570-0232(02)00431-2

1. Introduction

1.1. Tartrate-resistant acid phosphatase (TRAP) in patho-physiology of bone resorption

Bone is a dynamic tissue that constantly undergoes remodeling in which a coupled process of bone formation and resorption continues throughout life. During early life, bone formation exceeds bone resorption with a net increase in bone mass, while late in life bone resorption exceeds bone formation with a net loss of bone. Imbalance in bone formation and resorption can lead to various pathological conditions of bone such as osteoporosis, osteopetrosis, Paget's disease of the bone and hypercalcemia associated with malignant tumors [1]. The two cell types involved in bone remodeling are the osteoblast, engaged in bone formation, and the osteoclast, the essential cell for bone resorption [1,2]. Mature osteoclasts are multinucleated giant cells that express high levels of TRAP, the most noticeable feature of the osteoclast. Situated on the endosteal bone surfaces, these cells are polarized, furnished with a ruffled border surrounded by the sealing zone on their base, the basolateral membrane and the apical functional secretory domain [3,4].

The role of osteoclasts is to degrade mineralized bone matrix. This involves dissolution of crystalline hydroxyapatite and proteolytic cleavage of the organic matrix. Dissolution of mineral is achieved by secretion of HCl via the action of ATP-dependent vacuolar proton pumps across the ruffled border membrane as well as in intracellular vacuoles [5–7]. Several proteolytic enzymes such as lysosomal cysteine proteinases and matrix metalloproteinases degrade the collagen-rich bone matrix. Degradation products are removed from the resorption lacuna and transported through a transcytotic vesicular pathway from the ruffled border to the functional secretory domain, a site of exocytosis [8,9]. This transcytotic route allows osteoclasts to remove large amounts of matrix-degradation products without losing their tight attachment to underlying bone [4].

TRAP, also known as uteroferrin [10] or purple acid phosphatase [11], was first demonstrated by cytochemical staining of hairy cells of leukemic reticuloendotheliosis [12] and represented an acid phosphatase isoenzyme type 5 by electrophoresis [13]. Since the demonstration that osteoclastic acid phosphatase resists inhibition by tartrate [14–16], TRAP has been widely used as a cytochemical marker of the osteoclasts and their precursors for many years. TRAP has been also demonstrated in lung and spleen macrophages [17], and spleen cells in Gaucher's disease [18]. More recent studies using sensitive immunocytochemical and RT-PCR techniques have shown that TRAP is expressed in diverse tissues harboring cells of bone marrow origin, including dendritic cells, activated macrophages and cells belonging to the osteoclast/macrophage lineage [19–21], however, osteoclasts are still the most major cells that express TRAP.

The bone expresses the highest level of TRAP among various tissues examined [22]. In bone, TRAP is primarily demonstrated in multinucleated osteoclasts and mononuclear osteoclast precursors [16,23]. Both TRAP and TR-ATPase (a TRAP having substrate preference for ATP) stained osteoclasts by immunocytochemical methods, the latter being more specifically localized to the osteoclast and to the ruffled borders [24,25]. By biochemical measurement, the bone had the highest specific activity for both TRAP and TR-ATPase [25]. The osteoclastic TRAP may be appropriately described as TR-ATPase, which is a type 5 TRAP having similar physicochemical properties and strong relatedness in the N-terminal amino acid sequence to TRAP expressed in other tissues [26]. Purified human bone TRAP was also a TR-ATPase, similar to that isolated from rat bone [27]. By in situ hybridization technique, it has been shown that osteoclasts are the ones that express significant TRAP mRNA in the bone [22]. Thus, it is generally agreed that osteoclasts are the major cell type that synthesizes TRAP in bone. Unexplained TRAP activity, however, has been reported in osteoblasts and osteocytes of growing rat bones [28,29] and transiently in the medullary bone matrix of egg-laying birds [30].

Electron microscopic studies of osteoclasts confirmed TRAP in lysosomes, vacuoles, extracellular channels and channel expansions in the ruffled border, and cell–bone interspace [31,32]. Acid phosphatase [31], but not TRAP [32] was found in Golgi apparatus. Within the osteoclast cytoplasm, TRAP is localized mainly in lysosomes and some of these lysosomes have been observed to empty their con-

tents into larger, endocytic vesicles containing resorbed material [33], implying its role in intracellular bone degradation.

Recently, TRAP has received growing attention due to the proposed functional involvement in bone resorption [34]. A blocking antibody to porcine uteroferrin inhibited both the enzyme activity and bone resorbing activity of osteoclasts in vitro [35]. Mice lacking TRAP showed disrupted endochondral ossification and mild osteopetrosis [36], while transgenic mice over-expressing TRAP exhibited an increased rate of bone turnover and mild osteoporosis [37]. These studies indicate that TRAP is required for normal bone development and maintenance. The physiological substrates for TRAP have not been identified yet. The candidates are phosphorylated tyrosine [38,39], sugar-phosphates as ATP [40], mannose-6-phosphates [41] and bone matrix phosphoproteins, such as osteopontin (OPN) [42]. Studies of lysosomal acid phosphatase (LAP) and TRAP double deficient mice suggest TRAP is involved in processing of bone matrix phosphoproteins including OPN [43]. Halleen et al. proposed another possible function of TRAP [44]. TRAP can generate highly destructive reactive oxygen species (ROS) capable of destroying collagen and other proteins. As TRAP is co-localized in the transcytotic vesicles transporting matrix degradation product, it is suggested that TRAP facilitates fragmentation of endocytosed material in a specific sub-cellular compartment [44].

The idea to use TRAP as an indicator of bone resorption came from a study by Minkin which showed a release of TRAP and other acid phosphatases from cultured bones that were stimulated by parathyroid hormone or $1,25(OH)_2D_3$ [45]. TRAP is soluble and will be secreted by the osteoclast [26]. In fact, TRAP is present in the serum, and particularly TRAP 5b which has been shown to be identical to osteoclast TRAP [46,47] is regarded as a marker of osteoclastic bone resorption in the serum by many investigators [27,46–52]. This important topic will be further discussed in the following sections. The mechanism involved in TRAP secretion from osteoclasts is not well known, but as discussed above, TRAP is considered to be transported through the osteoclast in the transcytotic vesicles together with bone degradation product and probably secreted through the functional secretory domain [44]. Thus, TRAP, known for many years, is now emerging as an important biomarker in bone pathology, not only for histochemical identification of osteoclasts but also as a serum marker of osteoclast activity and bone turnover.

1.2. Analytical aspects of TRAP

1.2.1. TRAP gene structure and function

The cDNA sequences of TRAP 5 enzymes from different species and organs all indicate that these enzymes are translated as monomeric polypeptides of 35–37 kDa in human [53], rat [22] and pig [54]. TRAP gene is one single gene [55] in chromosome 15 [56] or 19 in human [53]. Alternative splicing or post-transcriptional modification would occur in TRAP RNA [10] or enzyme [57].

The function of TRAP 5 was investigated by targeted disruption of the gene in mice (TRAP-gene-knockout mice). Animals homozygous for the null TRAP 5 allele had progressive foreshortening and deformity of the long bones and axial skeleton but apparently normal tooth eruption and skull plate development, indicating a role for TRAP 5 in endochondral ossification. These findings show that a mild osteopetrosis is induced by the intrinsic defect of osteoclastic modeling activity, which was confirmed in the resorption pit assay in vitro [36]. The promoter region of TRAP gene contains several transcription factors, e.g. AP-1, PU.1, M-box and interferon regulatory factor element (IRF-E) regions. Matsumoto et al. [58] showed in murine cells that Pip (PU.1-interacting protein; IRF-4) that was induced by RANKL (receptor activator of NF-κB ligand) bound to IRF-E and regulates synergistically with *microphthalmia* transcription factor (MITF that was bound to M-box gene) to induce the promoter activity of TRAP gene. Luchin et al. [59] reported that PU.1 or MITF alone caused only 4–5-fold activation of TRAP gene and that the two transcription factors induced 20-fold activation synergistically when applied simultaneously. Comparison of the human TRAP 5′-flanking region with mouse TRAP and uteroferrin revealed 41% and 47% homology, respectively. This suggests that regulation of human TRAP gene expression may differ from that of the murine TRAP gene [60]. Furthermore, Reddy et al.

[61] reported a novel iron/hemin-responsive transcriptional regulatory mechanism in human cells.

Recombinant human and rat TRAP 5s were constructed using a baculovirus expression system by Hayman et al. [11] and Kaija et al. [62], respectively. Recombinant TRAP was purified and utilized to prepare monoclonal antibodies [63]. Marshall et al. [64] purified the recombinant human and mouse TRAP 5 from baculovirus-infected insect cells. The specific activity of the mouse enzyme increased threefold upon nicking.

1.2.2. Physicochemical characters of TRAP

The physicochemical characteristics of mammalian TRAP were described precisely by Oddie et al. [34]. The isolated TRAPs were found to be glycoproteins of 30–40 kDa molecular masses. The amino acid sequence of human placental TRAP, rat bone TRAP, bovine spleen TRAP and porcine spleen TRAP are highly conserved [10,22,53,65,66]. The glycoprotein structure of human bone TRAP was analyzed by using various lectins [27]. TRAP contains only N-linked high-mannose type oligosaccharide composed of five or six mannose residues and two *N*-acetylglucosamine residues. Essentially the same results were obtained with porcine uteroferrin [67,68]. Both TRAP and uteroferrin have comparable basic isoelectropoints (p*I* 8.5–9.5) [52,55]. Using acid native electrophoresis, TRAP 5 was separated into 5a and 5b in human serum. The optimal pH of TRAP 5a and 5b is 4.9–5.5 and 5.7–6.0 when *p*-nitrophenyl phosphate (pNPP) was used as substrate, respectively [47,52,69]. The difference in electrophoretic behavior between 5a and 5b is due to the fact that sialic acid binds to 5a isozyme but not to 5b isozyme [70]. The origin of TRAP 5a is not known so far [47,71] and most of 5b is believed to be produced in osteoclasts [50].

TRAP consists of two subunits of M_w 23 and 16 kDa in disulfide linkage in the rat [72]. Cleavage with papain resulted in complete activation and conferred similar properties to those of the bone purple acid phosphatase variant with regard to pH optimum (5.5–6.0) [73]. The physiological significance of the limited proteolysis of TRAP 5 is not quite clear. The post-translational cleavage might be an artifact of the purification procedure [74]. It is shown that three cysteine residues are found in the sequence of the rat enzyme, and two of these, Cys 163 and Cys 221, are positioned such that they might form a disulfide bond [73]. However, there was no indication of the formation of a disulfide bond in the crystals. Both of the papers [74,75] concerning crystal structure reported that TRAP has β–α–β–α–β motif structure and di-iron center Fe(III)–Fe(II).

1.2.3. Analytical methods

Disk gel electrophoresis would be the best way to assess the TRAP 5 and other acid phosphatases [70,76]. The TRAP isoenzymes have long been analyzed by electrophoresis [77], CM-Sepharose column chromatography [69] and isoelectric focusing [78]. TRAP was named as ''type 5 acid phosphatase'' based on its fast electrophoretic mobility toward the cathode on acidic acrylamide gel [12]. The band 5 was further separated into 5a and 5b by electrophoresis [69]. TRAP 5b was thought to be derived from osteoclasts and TRAP 5a from some other cells [70]. Serum TRAP 5b assays for diagnosis of bone diseases have been published recently. Nakanishi et al. [79] developed a kinetic assay method for detecting band 5b TRAP activity using an inhibitor for band 5a.

Column chromatography is not usually applied to analyze TRAP 5 but to prepare the enzyme. A number of researchers used cation-exchange resin, gel chromatography and affinity columns [69,80,81]. These analytical methods require a large amount of serum to detect TRAP activity [82]. Thus, they could not routinely be utilized for diagnosis of the above-mentioned diseases and other bone metabolic diseases. Recently a novel heparin column analysis of TRAP has been developed which improved separation of TRAP 5b from erythrocyte TRAP and TRAP 5a using only small amounts of serum [52].

Recently, an immunological method and a colorimetric method were combined together to assess the TRAP 5 or TRAP 5b for the purpose of mass screening of serum samples [47]. Monoclonal TRAP 5 antibodies so far prepared cannot distinguish 5a and 5b [46]. One should pay attention to the fact that TRAP 5b molecules are inactivated and degraded into fragments before they are removed from the circulation. A two-site antibody method may detect these peptides. After trapping antigen by monoclonal antibody, the trapped TRAP was tested

for its enzyme activity [47,51]. According to this method, degraded TRAPs do not disturb the estimation of TRAP activity.

1.3. Clinical chemistry application

Osteoclasts secrete TRAP 5 into the circulation. Early enzyme assays for TRAP 5 were disturbed by the presence of other acid phosphatases that were derived from erythrocyte and platelet cells [83,84]. High blood levels of TRAP 5 are usually associated with active bone remodeling [48,63]. Increased serum levels of TRAP 5b are observed during normal physiological bone growth among healthy children [52,85]. These findings provide evidence that TRAP 5 is involved in normal bone homeostasis in vivo. Sensitive and specific serum markers for bone formation and resorption are essential for clinical assessment for bone metabolism.

Specific estimation of TRAP 5b would provide a useful parameter in the evaluation of bone loss even though TRAP 5 isoforms' expression may be variable in different diseases. Elevated serum TRAP levels have been detected in diseases characterized by increased bone resorption; Paget's disease of the bone [86], Gaucher's disease [70], hairy cell leukemia [87,88], hemodialysis [89,90], primary hyperparathyroidism [91], metastatic malignancies involving bone resorption [92], multiple myeloma [93] and bilateral ovariectomized women [94]. Postmenopausal women have higher levels of serum TRAP 5b than post-menopausal women on estrogen replacement therapy [47]. It is reported that there is little sex difference in the TRAP activity of children, younger than 10 years of age [95,96]. A significant positive correlation was found between the bone isoenzyme of bone alkaline phosphatase and plasma TRAP for normal persons, irrespective of age and sex from 10 to 80 [97].

2. Methods of enzyme assays in serum

2.1. Methods applied up to date

Withold [98] descried that TRAP (as determined by the available methods in 1996) cannot be recommended as a routine tool for assessment of bone resorption. Nearly the same opinion was proposed by Eyre [99] in 1997 and Swaminathan [100] in 2001. The full validity of these TRAP assays and their value in the management of osteoporosis remain to be evaluated. Paglia et al. [101] reported that bone turnover after a short period of steroid therapy in elderly men was assessed using TRAP activity that measured all TRAPs in the serum. They found no difference between patients and controls in the mean serum levels of TRAP, whereas mean serum levels of C-terminal telopeptides of type I collagen showed statistical difference. Because the serum contains certain other cell types of TRAP, total TRAP would be an unreliable marker to assess bone turnover of these patients. For decades, however, total TRAP activity has been regarded as a bone resorption marker in the serum [94,96,102]. In the serum, total TRAP activities can be derived not only from bone but also from erythrocytes and platelets. The increase in band 5a has never been observed in any pathologic conditions when analyzed using native electrophoresis at pH 4 [103]. Takahashi and Inoue [104] stated that not only urinary hydroxyproline but also serum TRAP lacked the sensitivity and specificity to bone resorption. Moreover, serum TRAP was shown to be a poor indicator of bone turnover in response to hormone replacement therapy [105]. Assay of total TRAP showed no appreciable differences before and after menopause. Thus, deoxypyridinoline or crosslinked N-telopeptide (NTx) are the current choice of bone resorption markers [100].

Lau et al. [48] improved a spectrophotometric assay of TRAP activity in human serum. Human serum contains a dialyzable, mixed-type noncompetitive inhibitor(s) of TRAP activity, the effects of which on the assay were substantially lessened by diluting the serum sample with buffer before assay and increasing the substrate concentration. They showed for the first time that hemolysis released into serum a significant amount of TRAP activity from erythrocytes, which can be inactivated by incubating the serum at 37 °C for 1 h before assay. Lau et al. [48] claimed that TRAP activity in the serum could be used clinically as a marker of bone metabolism. Enzyme activity was measured in 84 normal subjects and in 109 patients with common metabolic bone diseases according to Lau's procedure. Postmenopausal women showed significantly higher

mean values of serum TRAP activity (10.5±2.0 U/l; $P<0.01$) compared with mean values in pre-menopausal women (8.45±1.8 U/l). Increases in activity have also been demonstrated in patients with hyperparathyroidism, Paget's disease, bone malignancy, chronic maintenance hemodialysis and in children. These results suggested that the measurement of serum TRAP activity can be clinically useful to assess bone turnover [106].

2.2. The latest assay methods

Halleen et al. [27] purified band 5 TRAP from human bone that had molecular mass of 32 kDa and optimum-pH of 5.7 and contained no sialic acid. It has been shown that this 5b is composed of only high-mannose type carbohydrates [70]. A polyclonal antibody was used to assess the TRAP concentration in serum by a competitive fluorescence immunoassay and stained human tissues such as bone and lungs [27]. The bone and lungs were positive on osteoclasts and alveolar macrophage cells, respectively. Band 5 was not found in normal human spleen, whereas it was the dominant band in the leukemic spleen as analyzed by Lam et al. [107]. Thus, one can expect that the band 5b TRAP may be derived from bone and alveolar macrophages. Specific measurement of TRAP 5b in the serum would be much more useful for the estimation of bone resorbing activity than that of total TRAP activity.

Halleen et al. [108] developed monoclonal anti-TRAP antibodies, OA1 and J1B, using purified human bone TRAP as antigen. The epitopes are located far away from each other on the antigen. They developed a direct two-site fluoroimmunoassay and showed that TRAP existed in fresh serum as a complex that was unstable (>250 kDa) in storage at 4 °C for 24 h and turned to general TRAP (30 kDa). Pre-menopausal women had 13.1 μg/l TRAP in their sera according to the direct immunoassay [108], while it was estimated as 112.2 μg/l according to the competitive assays [27]. This means the 90% of TRAP in the circulation may be in the fragmented forms and only 10% of TRAP is in native form. Halleen et al. [47] showed that the Western analysis of SDS electrophoresis for purified human TRAP revealed a sharp band on the membrane with O1A antibody. O1A antibody could react not only with native TRAP but also with denatured form. There was a discrepancy between O1A specificity for tissue staining [109] and Western blotting. Chamberlain et al. [63] using two types of anti-recombinant TRAP (2H1 and 4E6) antibodies showed similar levels of TRAP in the serum. They used a new method for epitope stoichiometry by surface plasmon resonance technology for recombinant enzyme protein. When a new highly specific anti-TRAP 5b antibody is found in future, low concentrations of TRAP in serum can be assessed accurately by this method.

The difference in optimal pH for pNPP of TRAP is utilized to assess the 5b activity. Acid phosphatase activity of 5b was measured at pH 6.2. Since the optimal pH of 5a was lower than that of 5b by about one pH [47,69], the activity of 5a does not disturb the measurement of 5b activity at pH 6. The ratio of 5a to 5b is not always constant, therefore, if 5a is quite high, the assessment of 5b activity may be overestimated by this method [110].

A kinetic method was developed by Nakanishi et al. [85]. They measured the activity of tartrate-resistant and fluoride-sensitive acid phosphatase by using 2,6-dichloro-4-acetylphenylphosphate as substrate in the presence of polybrene at pH 6.2. They showed that the fluoride-sensitive TRAP corresponded to band 5 and that non-sensitive TRAP was derived from erythrocytes, platelets and leukocytes. Heparin inhibited band 5a TRAP activity but had no effect on band 5b TRAP in serum or bone extract in their system at pH 6.6 [79]. They compared the TRAP 5b activity that was measured in the presence of heparin at pH 6.6 with TRAP 5 activity that was measured in the presence of polybrene at pH 6.2, using healthy children ($n=22$) and adults' serum ($n=28$). The correlation coefficient between band 5b and TRAP 5 was 0.97. The band 5a and other unknown TRAP occupied 10 U/l constantly for all their healthy samples.

Janckila et al. [82] changed the substrate of TRAP from pNPP to N-ASBI phosphate. pNPP was hydrolyzed efficiently by erythrocyte and platelet TRAPs, whereas N-ASBI-phosphate was a poor substrate for TRAPs except TRAP 5. N-ASBI-phosphate was selectively hydrolyzed by TRAP 5b that was separated by cation-exchange chromatography. Isoform 5a shows very low activity in normal subjects and patients with rheumatic disease and rheumatoid

arthritis, but high in those with end-stage renal disease. In sera from patients with rheumatic disease, isoform 5b activity decreased to a half using N-ASBI phosphate. There may be some difference in substrate specificity among TRAP 5b from different diseases. The full validity of this phenomenon needs to be further evaluated.

2.3. Stability of TRAP

TRAP has been reported to be labile by several investigators. Lieberherr et al. [111] prepared TRAP containing cytoplasmic extract from newborn-rat calvaria. The enzyme was unstable in the presence of pNPP at 37 °C in 0.1 *M* acetate buffer at pH 5.4. Minkin [45] purified TRAP from organ cultures of newborn mouse calvaria. The considerable discrepancy between the total activity of enzyme in the non-cultured controls and the cultured bone and their media was attributed to the instability of the enzyme. After Stepan et al. [94] studied the activity of plasma TRAP using spectrophotometry, they recommended that hemolysis and blood coagulation should be prevented during plasma preparation. Because the enzyme is unstable, the assay should be performed immediately after sampling. Recently, Brehme et al. [112] reported that α2-macroglobulin trapped TRAP, the complex of which was referred to as high molecular mass TRAP (HMW TRAP 700 kDa). The HMW complex decreases the reactivity of TRAP to monoclonal-antibodies and TRAP activity. The HMW TRAP was found in certain eluted fractions of child serum that contained the highest TRAP activity in the life period using a size exclusion chromatography. The ratio of HMW TRAP to the normal TRAP is rather small but the occurrence of the former should be taken into consideration. The α2-macroglobulin can trap a variety of proteins in the serum by covalent and noncovalent mechanisms [113]. The ratio of TRAP to all trapped proteins against α2-macrogloblin in adult serum is smaller than in the child serum. Adult serum may not show the same ratio of HMW TRAP/TRAP as child serum. Because there is no evidence that α2-macroglobulin can trap TRAP 5b exclusively, the presence of HMW may lead to an erroneous conclusion although the effect may not be so large in TRAP 5b estimation.

Lau et al. [48] preincubated sera at 37 °C for 1 h before enzyme assay in order to eliminate the TRAP activity from erythrocytes and platelets [106]. They showed TRAP 5 was stable at 37 °C for several hours at pH 5.5. Because fluoride ion inhibits TRAP 5 nearly completely but not erythrocytic and platelet TRAP activities, Nakanishi et al. [85] roughly estimated TRAP 5b activity by the difference between the total activity and the fluoride-inhibited activity. They reported that the TRAP 5b activity in serum was stable for at least 12 h at room temperature and for 1 year at −80 °C. Halleen et al. [47] published an improved assay method of TRAP 5b using monoclonal anti-TRAP 5. They reported that serum TRAP 5b activity was rather stable. After 8 h incubation of serum at 25 °C, the immunoassay detected more than 90% of the amount that was detected before starting the incubation period. Repeated freezing/thawing cycles up to six times resulted in no changes in the activity of TRAP 5b detected. Halleen and Ranta [71] pointed out again that active serum TRAP 5b was quite stable and that no instability problems were associated with the assay.

3. Chromatographic methods

3.1. Ion-exchange chromatography

Cation-exchanger chromatography has been used as an extremely powerful tool to purify TRAP for a long time, because the p*I* of TRAP is 8.5–9.5. Starting materials were derived from human bone [81], rat bone [80,114], hairy cell leukemia spleen [107], Gaucher's disease spleen [70] and recombinant proteins [62,72]. All samples were adjusted to pH 5 and supernatants were applied to cation-exchangers, e.g. CM-cellulose [80,107,114,115], CM-Sepharose [116], ZetaChrom SP 100 [81], Sepharose S fast flow [46] and Mono-S [117]. TRAP was eluted as one peak by high concentration of sodium chloride. Anderson et al. [114] showed two types of acid phosphatase; one was a weakly bound form, E1 and the other was a strongly bound form, E2. E2 was not affected by tartrate but E1 was almost completely inhibited by it. Another experiment showed the TRAP peak was eluted as one peak that may correspond to TRAP 5b.

Acid phosphatase activity was divided into four major peaks in CM-52. The last peak was TRAP 5 that was confirmed by electrophoresis. Kraenzlin et al. [116] prepared TRAP 5b from hairy cell leukemic spleen by CM-Sepharose. The elution profile showed that there were at least three acid phosphatase isoenzymes in the extracts of hairy cell leukemic spleen. The last two of them were resistant to tartrate inhibition. Acidic native polyacrylamide gel electrophoresis indicated that only peak III showed electrophoretic mobility as serum TRAP 5b did.

In human serum, cation-exchange chromatography successfully separated TRAP 5a from 5b [46,69,82]. The sample volume was between 250 and 300 ml for Sepharose S column and 1 l for the CM-Sepharose column. All sera were adjusted to pH 5 and they formed some precipitants. Thus, these cation-exchanger methods cannot be applied to estimate the TRAP 5b with a serum obtained from one person. This method showed that the ratio of 5a/5b TRAP activity on chromatography was not equal to the ratio obtained by native disk gel electrophoresis pattern [110].

3.2. Affinity chromatography

Heparin column-bound TRAP in human cord serum was separated into three peaks of TRAP activity when eluted with a linear gradient of sodium chloride (Fig. 1) [52]. The last peak corresponded to TRAP 5b which was first named according to its electrophoretic mobility [69] and was considered to be derived from osteoclasts [108]. The second peak (peak II) was found to be TRAP 5a. The height of the last peak (peak III) varied from age to age in healthy persons (Fig. 2). The peak ratio (2.2) in adult serum of 5a to 5b (Fig. 2) when analyzed by heparin column was similar to the ratio obtained by electrophoretic assay (>2). The activity of 5a is higher than that of 5b using pNPP TRAP assay. The sera of five adult patients with end-stage renal disease were analyzed for their TRAP activity after separation by heparin column. All 5b peaks of the patients were higher than the healthy adult (Fig. 3). Fig. 1 shows that almost all TRAP activity of the unbound fraction was inhibited by fluoride ion [118]. This means there exists some TRAP other than erythrocyte and platelet TRAP.

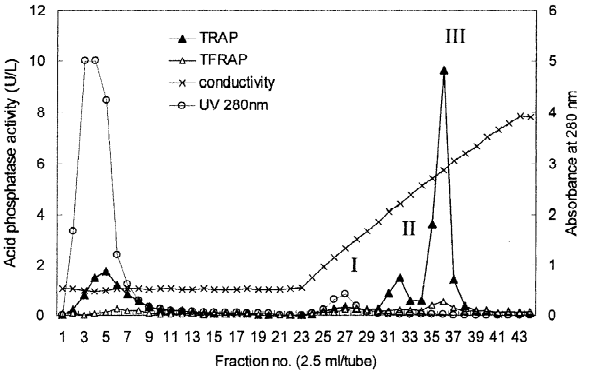

Fig. 1. The chromatographic separation of acid phosphatase activity in cord serum on a 5 ml of heparin column. Cord serum was dialyzed against the equilibrium buffer; 20 m*M* Tris–HCl, 0.1 *M* NaCl pH 7.2, and 6 ml were applied to the heparin column. The TRAPs were eluted by a NaCl linear gradient from 0.1 *M* to 1.05 *M* (star) and fractionated, 2.5 ml each. Between fraction 20 and 21, the column was washed with 72 volumes of the equilibrium buffer. The protein was monitored using UV at 280 nm (open circle). The activity of eluted TRAP was assayed in 100 m*M* citrate buffer (pH 5.5) that contained 50 m*M* *p*-nitrophenyl phosphate and 40 m*M* sodium (+) tartrate, with (open triangle) or without (closed triangle) 25 m*M* of sodium fluoride. All fractions were 2.5 ml each per tube.

Con A-Sepharose was also used for purification of TRAP. The eluted TRAP showed a very sharp peak and was purified 2500-fold [80]. This column meth-

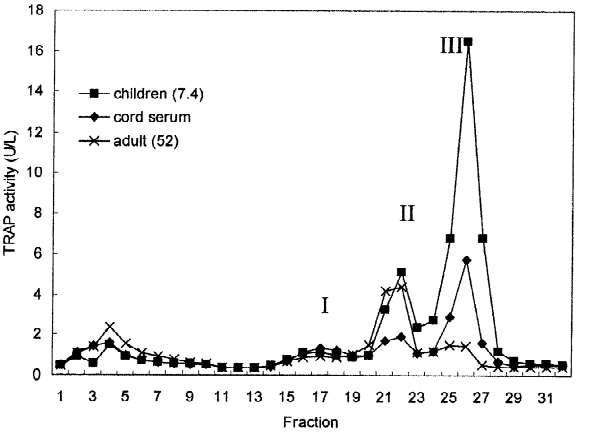

Fig. 2. The elution profile on the heparin column of the cord, child and adult serum. Eight ml of each serum were applied on the heparin column. Unbound TRAP was fractionated, 5 ml each, from fractions numbered 1 to 10. After washing the column, bound TRAPs were fractionated, 2.5 ml each, from 11 to 32. The numbers in parentheses indicate the age.

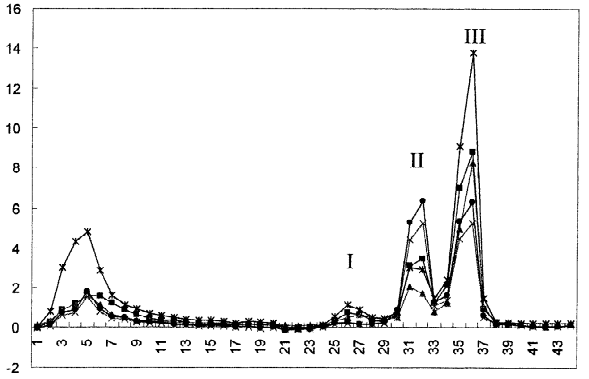

Fig. 3. The elution profile on the heparin column of the end-stage renal disease sera. Six ml of serum from five end-stage renal disease patients each were applied on the heparin column. Unbound TRAP was fractionated, 5 ml each, from fractions numbered 1 to 10, and after washing the column, the bound TRAPs were fractionated, 2.5 ml each.

od can be used for the assay of TRAP in the serum with mannose gradient. However, they eluted the TRAP from the ConA column without continual flow of mannose gradient.

4. Electromigration methods

4.1. Native electrophoresis

TRAP was first recognized as one out of seven distinct bands separable by polyacrylamide gel electrophoresis at 4 °C [12]. TRAP is often called band 5, or isoenzyme 5. A discontinuous acid native plate gel showed good separation for band 5 [112]. Lam et al. [70] showed the electrophoretic difference of 5a and 5b using fractions that were separated by CM cellulose and obtained similar results as shown in Fig. 4 [52]. Usually, a disk gel separates band 5 from other acid phosphatases in the serum, whereas band 5 cannot be separated into 5a and 5b distinctly [119]. However, disk gel electrophoresis separated and stained TRAP 5a and 5b clearly enough when long gel was applied [110]. The ratio of 5a to 5b was 7:2 for normal adult serum. This ratio may reflect the condition of the patient. In principle, the band comes out as an accumulated dye that is coupled with the substrate. The band volume depends upon the amount of the enzyme and electrophoretic conditions. The ratio of 5a to 5b was less than unity (5b is greater than 5a) for healthy pooled serum with a cation-exchange chromatography [46]. However, Janckila et al. [82] reported that N-ASBI-P was a preferred substrate for 5b when staining the electrophoresis gel in general. There is a possibility that 5a band volume becomes larger than usual. No one can explain the cause of this discrepancy.

The electrophoretic method is not so complicated and a large sample volume is not required, i.e. about 50 μl. Thus, this method will be used to assess TRAP 5b in the future. One problem may be coupling dye that possibly inhibits the enzyme activity with accumulation for a long reaction time. Another drawback is to determine the enzyme activity by the coupling dye that is not transparent. It will not be reproducible.

4.2. Isoelectrophoresis

Cultured leukemia–lymphoma cells were analyzed for the expression of TRAP separated by isoelectric focusing (ampholyte pH 2–11) by Drexler [78]. TRAP was stained as one band of activity. These cells may not have 5a as bone but only 5b. Cheung [120] used a Rotofor preparative isoelectro-focusing unit, which contained 1% ampholyte (pH 3–10) to prepare the TRAP for polyclonal antigen. TRAP was focused around pH 7 as one peak. Before this purification step, they used CM-Sepharose to separate other ingredients. TRAP 5a may be decreased at this step. Basically, the isoelectrophoretic method could be applied to analyze TRAP in serum and tissues.

A major component of cord TRAP moved to an alkaline pH close to cathode and a minor component was focused at neutral pH. The main bands of child and adult TRAP moved to a comparable position as the cord TRAP. The p*I* of adult bone TRAP was estimated to be ~9 by Ampholine gel [55]. The p*I* of cord TRAP was not different from that of the adult TRAP (Fig. 5) [52]. Sialic acid molecules attached to the 5a polypeptide decrease the p*I* of 5a and/or shift a different three-dimensional structure from 5b [70]. These changes of sialic acid contents induce a slightly different binding property to the heparin column as peak II and III (as shown in Fig. 1).

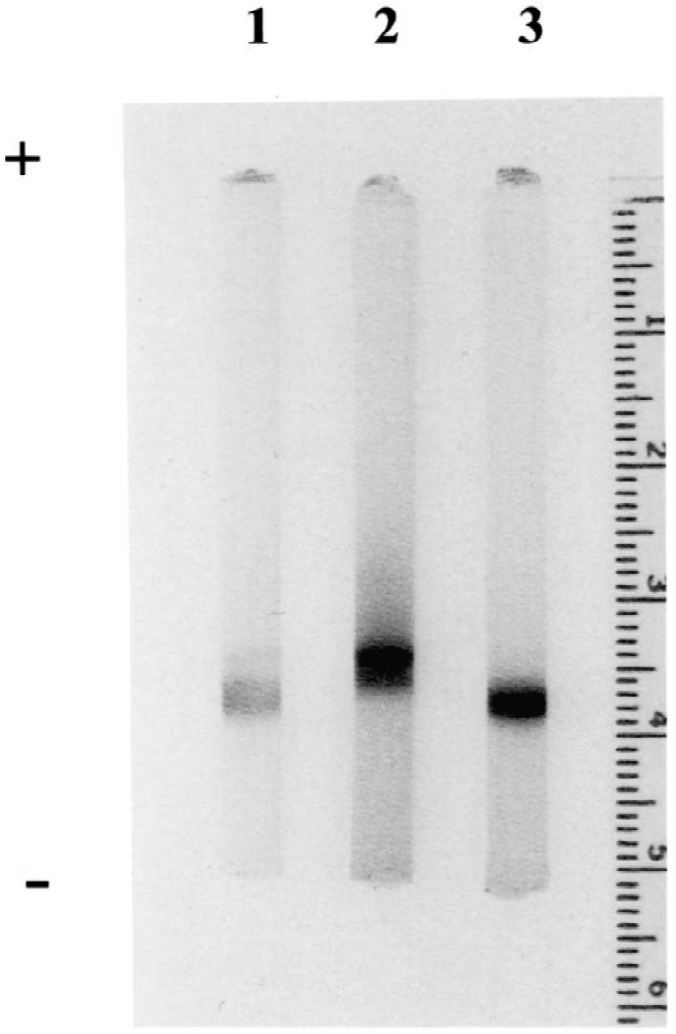

Fig. 4. Native electrophoresis using polyacrylamide disk gel. Electrophoretic analyses of cord serum TRAP separated on heparin column. Disk 1 is peak I of the heparin column chromatography. Disk 2 is peak II and disk 3 is peak III. The activity staining of TRAP was performed using naphthol ASTR phosphate as the substrate, hexazotized pararosaniline as a coupling dye according to Lee et al. [125]. The method of native electrophoresis was performed according to Lam et al. [77].

5. Critical evaluation of biological relevance of the analytical results and conclusions

As discussed above, TRAP 5b is a prominent product of osteoclasts. Osteoclast TRAP is engaged in bone matrix degradation in extracellular and intracellular manners, transported through the cell by a transcytotic pathway, and is likely secreted from the osteoclast with bone degradation products. Serum TRAP 5b levels would reflect the status of bone resorption; however, TRAP 5b is not the only TRAP enzyme in the serum. TRAP from erythrocytes and platelets are also present in the serum as the enzyme is released from the cells during blood collection and serum separation, and it can constitute a source of error in serum TRAP measurement. Osteoclasts are the only major cell type that synthesizes TRAP in bone as shown by histochemical, immunocytochemi-

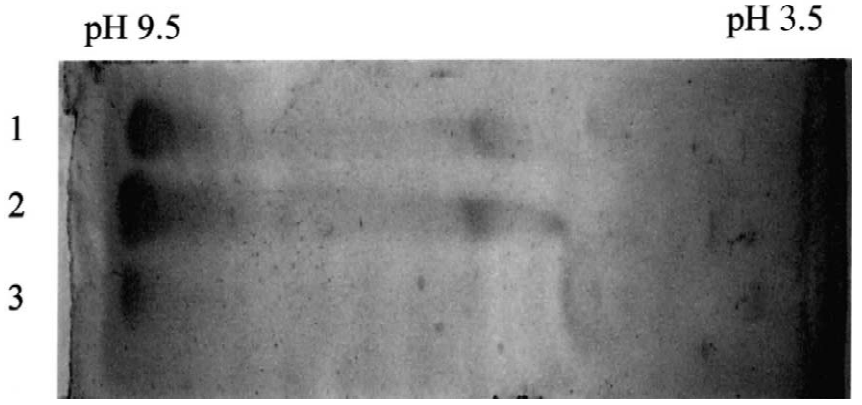

Fig. 5. Isoelectrophoresis using polyacrylamide plate gel. Isoelectric focusing of peak III of TRAP. Lane 1, cord sera; lane 2, child sera; lane 3, adult sera. The electrophoresis was performed at 1500 V for 3.5 h following the manufacturer's description using Ampholine PGAplates (pH 3.5–9.5) (Amersham Pharmacia Biotech). The activity staining of TRAP was performed according to Lee et al. [125].

cal and in situ hybridization studies. Therefore, only TRAP 5b should be quantified in the serum to accurately assess bone metabolism.

Total TRAP may serve as a bone resorption marker only in children [95,121]. Because the TRAP 5b peak predominates in the child serum, the total TRAP activity would roughly reflect bone resorption; whereas in adult, the TRAP 5b peak is much lower than the 5a, thus, the total TRAP would not necessarily reflect the bone activity. This is the reason why TRAP 5b should be analyzed correctly for evaluation of bone resorption. Nakanishi et al. [79] claimed that 5b activity could be determined in the presence of heparin at pH 6.6. They showed a good correlation between TRAP 5b and total TRAP 5. However, they neglected unbound TRAP; total TRAP contained higher than 10 U/l activities at zero concentration of TRAP 5b.

On the heparin column, unbound fractions contained three types of acid phosphatase; i.e. acid phosphatase, TRAP and tartrate and fluoride resistant acid phosphatase (TFRAP) activity. The ratio of TFRAP to TRAP was very low (1/5) in this peak. The ratio of acid phosphatase to TRAP was near unity. It means that the unbound peak contains not only erythrocyte TRAP but also other TRAPs that may overlap with an unmoved fraction in a native electrophoretic disk-gel from cultured bone homogenates using α-naphthyl phosphate staining by Minkin [45]. As an inevitable consequence of the hemolysate, total TRAP would be increased. The analysis done by our heparin column method was not affected by hemolysate as shown in Fig. 1. Our results show that heparinized sera are not suitable for the estimation of TRAP activity, because heparin binds TRAP and partially inhibits TRAP activity [52]. TRAP 5b binds to the heparin column more strongly than 5a at pH 7.2. In our study pNPP was used for the substrate of TRAP at pH 5.5. TRAP 5b activity was inhibited ~10% with heparin (25 U/ml final). Nakanishi et al. [79] used 2,6-dichloro-4-acetylphenyl phosphate as the substrate and assayed at pH 6.6. The discrepancy in pH optima may have resulted from the difference in substrates used.

Halleen et al. [47] showed that monoclonal antibody O1A captured TRAP 5 (both 5a and 5b). The optimal pH for TRAP 5b activity is 6.1, but at this pH, TRAP 5a has less than one fifth of its optimal activity at pH 5.0. On the basis of these findings, they measured TRAP 5b activity at pH 6.1 in order to capture 5b only while MAb O1A recognizes both TRAP 5a and 5b. In our experiments (Fig. 2), TRAP 5a activity in the sera of elderly persons was usually three or more times higher than the 5b activity. TRAP 5b in such sera might have been overestimated by their MAb O1A method that would capture both 5a and 5b. A specific anti-TRAP 5b antibody could be useful for the development of enzyme linked immunosorbent assay (ELISA) for TRAP assay but any attempt to obtain a highly specific

antibody has been without success to date [46,47,108,122,123].

One cannot neglect the possibility that some TRAP activity derived from alveolar macrophages is contaminating the serum [124]. It remains to be clarified to what extent 5b from the alveolar cells contribute to the total TRAP 5b in the serum in future.

References

[1] G.D. Roodman, Endocr. Rev. 17 (1996) 308.
[2] T. Suda, N. Takahashi, N. Udagawa, E. Jimi, M.T. Gillespie, T.J. Martin, Endocr. Rev. 20 (1999) 345.
[3] M.E. Holtrop, in: The Osteoclast, Bone, Vol. 2, CRC Press, 1991.
[4] H.K. Väänänen, H. Zhao, M. Mulari, J.M. Halleen, J. Cell Sci. 113 (Pt. 3) (2000) 377.
[5] R. Baron, L. Neff, D. Louvard, P.J. Courtoy, J. Cell Biol. 101 (1985) 2210.
[6] R. Baron, L. Neff, C. Roy, A. Boisvert, M. Caplan, Cell 46 (1986) 311.
[7] H.C. Blair, S.L. Teitelbaum, R. Ghiselli, S. Gluck, Science 245 (1989) 855.
[8] S.A. Nesbitt, M.A. Horton, Science 276 (1997) 266.
[9] J. Salo, P. Lehenkari, M. Mulari, K. Metsikko, H.K. Väänänen, Science 276 (1997) 270.
[10] P. Ling, R.M. Roberts, J. Biol. Chem. 268 (1993) 6896.
[11] A.R. Hayman, T.M. Cox, J. Biol. Chem. 269 (1994) 1294.
[12] C.Y. Li, L.T. Yam, K.W. Lam, J. Histochem. Cytochem. 18 (1970) 473.
[13] L.T. Yam, C.Y. Li, K.W. Lam, N. Engl. J. Med. 284 (1971) 357.
[14] L.E. Hammarström, J.S. Hanker, S.U. Toverud, Clin. Orthop. 78 (1971) 151.
[15] J.E. Wergedal, Proc. Soc. Exp. Biol. Med. 134 (1970) 244.
[16] F.P. van de Wijngaert, E.H. Burger, J. Histochem. Cytochem. 34 (1986) 1317.
[17] J. Schindelmeiser, P. Schewe, T. Zonka, D. Munstermann, Histochemistry 92 (1989) 81.
[18] C.M. Woodruff, H.R. Schumacher, M.C. Bilak, S.A. Stass, Am. J. Clin. Pathol. 71 (1979) 361.
[19] A.R. Hayman, A.J. Bune, T.M. Cox, J. Anat. 196 (Pt. 3) (2000) 433.
[20] A.R. Hayman, A.J. Bune, J.R. Bradley, J. Rashbass, T.M. Cox, J. Histochem. Cytochem. 48 (2000) 219.
[21] A.R. Hayman, P. Macary, P.J. Lehner, T.M. Cox, J. Histochem. Cytochem. 49 (2001) 675.
[22] B. Ek-Rylander, P. Bill, M. Norgard, S. Nilsson, G. Andersson, J. Biol. Chem. 266 (1991) 24684.
[23] G.N. Andersson, B. Ek-Rylander, L.E. Hammarström, S. Lindskog, S.U. Toverud, J. Histochem. Cytochem. 34 (1986) 293.
[24] G.N. Andersson, S.C. Marks Jr., J. Histochem. Cytochem. 37 (1989) 115.
[25] A. Lindunger, C.A. MacKay, B. Ek-Rylander, G. Andersson, S.C. Marks Jr., Bone Miner. 10 (1990) 109.
[26] B. Ek-Rylander, T. Bergman, G. Andersson, J. Bone Miner. Res. 6 (1991) 365.
[27] J. Halleen, T.A. Hentunen, J. Hellman, H.K. Väänänen, J. Bone Miner. Res. 11 (1996) 1444.
[28] P. Bianco, P. Ballanti, E. Bonucci, Calcif. Tissue Int. 43 (1988) 167.
[29] E. Bonucci, P. Mocetti, G. Silvestrini, P. Balianti, S. Zalzal, M. Fortin, A. Nanci, J. Electron Microsc. (Tokyo) 50 (2001) 333.
[30] T. Yamamoto, H. Nagai, J. Bone Miner. Res. 7 (1992) 1267.
[31] U. Lucht, Histochemie 28 (1971) 103.
[32] S.A. Clark, W.W. Ambrose, T.R. Anderson, R.S. Terrell, S.U. Toverud, J. Bone Miner. Res. 4 (1989) 399.
[33] F.P. Reinholt, S.M. Widholm, B. Ek-Rylander, G. Andersson, J. Bone Miner. Res. 5 (1990) 1055.
[34] G.W. Oddie, G. Schenk, N.Z. Angel, N. Walsh, L.W. Guddat, J. de Jersey, A.I. Cassady, S.E. Hamilton, D.A. Hume, Bone 27 (2000) 575.
[35] M. Zaidi, B. Moonga, D.W. Moss, I. MacIntyre, Biochem. Biophys. Res. Commun. 159 (1989) 68.
[36] A.R. Hayman, S.J. Jones, A. Boyde, D. Foster, W.H. Colledge, M.B. Carlton, M.J. Evans, T.M. Cox, Development 122 (1996) 3151.
[37] N.Z. Angel, N. Walsh, M.R. Forwood, M.C. Ostrowski, A.I. Cassady, D.A. Hume, J. Bone Miner. Res. 15 (2000) 103.
[38] J.M. Halleen, H. Kaija, J.J. Stepan, P. Vihko, H.K. Väänänen, Arch. Biochem. Biophys. 352 (1998) 97.
[39] K. Nash, M. Feldmuller, J. de Jersey, P. Alewood, S. Hamilton, Anal. Biochem. 213 (1993) 303.
[40] J.B. Vincent, B.A. Averill, FEBS Lett. 263 (1990) 265.
[41] R. Bresciani, K. Von Figura, Eur. J. Biochem. 238 (1996) 669.
[42] B. Ek-Rylander, M. Flores, M. Wendel, D. Heinegard, G. Andersson, J. Biol. Chem. 269 (1994) 14853.
[43] A. Suter, V. Everts, A. Boyde, S.J. Jones, R. Lullmann-Rauch, D. Hartmann, A.R. Hayman, T.M. Cox, M.J. Evans, T. Meister, K. von Figura, P. Saftig, Development 128 (2001) 4899.
[44] J.M. Halleen, S. Raisanen, J.J. Salo, S.V. Reddy, G.D. Roodman, T.A. Hentunen, P.P. Lehenkari, H. Kaija, P. Vihko, H.K. Väänänen, J. Biol. Chem. 274 (1999) 22907.
[45] C. Minkin, Calcif. Tissue Int. 34 (1982) 285.
[46] A.J. Janckila, K. Takahashi, S.Z. Sun, L.T. Yam, Clin. Chem. 47 (2001) 74.
[47] J.M. Halleen, S.L. Alatalo, H. Suominen, S. Cheng, A.J. Janckila, H.K. Väänänen, J. Bone Miner. Res. 15 (2000) 1337.
[48] K.H. Lau, T. Onishi, J.E. Wergedal, F.R. Singer, D.J. Baylink, Clin. Chem. 33 (1987) 458.
[49] H. Rico, L.F. Villa, Calcif. Tissue Int. 52 (1993) 149.
[50] C.P. Price, A. Kirwan, C. Vader, Clin. Chem. 41 (1995) 641.
[51] J.M. Halleen, S.L. Alatalo, A.J. Janckila, H.W. Woitge, M.J. Seibel, H.K. Väänänen, Clin. Chem. 47 (2001) 597.

[52] Y. Igarashi, M.Y. Lee, S. Matsuzaki, J. Chromatogr. B Biomed. Sci. Appl. 757 (2001) 269.
[53] D.K. Lord, N.C. Cross, M.A. Bevilacqua, S.H. Rider, P.A. Gorman, A.V. Groves, D.W. Moss, D. Sheer, T.M. Cox, Eur. J. Biochem. 189 (1990) 287.
[54] R.C. Simmen, V. Srinivas, R.M. Roberts, DNA 8 (1989) 543.
[55] H.G. Drexler, S.M. Gignac, Leukemia 8 (1994) 359.
[56] B.S. Allen, C.M. Ketcham, R.M. Roberts, H.S. Nick, H. Ostrer, Genomics 4 (1989) 597.
[57] A.R. Hayman, A.J. Dryden, T.J. Chambers, M.J. Warburton, Biochem. J. 277 (Pt. 3) (1991) 631.
[58] M. Matsumoto, K. Hisatake, Y. Nogi, M. Tsujimoto, J. Biol. Chem. 276 (2001) 33086.
[59] A. Luchin, S. Suchting, T. Merson, T.J. Rosol, D.A. Hume, A.I. Cassady, M.C. Ostrowski, J. Biol. Chem. 276 (2001) 36703.
[60] S.V. Reddy, N. Kuzhandaivelu, L.G. Acosta, G.D. Roodman, Bone 16 (1995) 587.
[61] S.V. Reddy, O. Alcantara, G.D. Roodman, D.H. Boldt, Blood 88 (1996) 2288.
[62] H. Kaija, J. Jia, Y. Lindqvist, G. Andersson, P. Vihko, J. Bone Miner. Res. 14 (1999) 424.
[63] P. Chamberlain, J. Compston, T.M. Cox, A.R. Hayman, R.C. Imrie, K. Reynolds, S.D. Holmes, Clin. Chem. 41 (1995) 1495.
[64] K. Marshall, K. Nash, G. Haussman, I. Cassady, D. Hume, J. de Jersey, S. Hamilton, Arch. Biochem. Biophys. 345 (1997) 230.
[65] C.M. Ketcham, R.M. Roberts, R.C. Simmen, H.S. Nick, J. Biol. Chem. 264 (1989) 557.
[66] D.F. Hunt, J.R. Yates III, J. Shabanowitz, N.Z. Zhu, T. Zirino, B.A. Averill, S.T. Daurat-Larroque, J.G. Shewale, R.M. Roberts, K. Brew, Biochem. Biophys. Res. Commun. 144 (1987) 1154.
[67] G.A. Baumbach, P.T. Saunders, F.W. Bazer, R.M. Roberts, Proc. Natl. Acad. Sci. USA 81 (1984) 2985.
[68] P.T. Saunders, R.H. Renegar, T.J. Raub, G.A. Baumbach, P.H. Atkinson, F.W. Bazer, R.M. Roberts, J. Biol. Chem. 260 (1985) 3658.
[69] W.K. Lam, D.T. Eastlund, C.Y. Li, L.T. Yam, Clin. Chem. 24 (1978) 1105.
[70] K.W. Lam, C.Y. Li, L.T. Yam, R.J. Desnick, Clin. Biochem. 14 (1981) 177.
[71] J.M. Halleen, R. Ranta, Eur. Clin. Lab. 20 (2001) 12.
[72] B. Ek-Rylander, T. Barkhem, J. Ljusberg, L. Ohman, K.K. Andersson, G. Andersson, Biochem. J. 321 (Pt. 2) (1997) 305.
[73] J. Ljusberg, B. Ek-Rylander, G. Andersson, Biochem. J. 343 (Pt. 1) (1999) 63.
[74] Y. Lindqvist, E. Johansson, H. Kaija, P. Vihko, G. Schneider, J. Mol. Biol. 291 (1999) 135.
[75] J. Uppenberg, F. Lindqvist, C. Svensson, B. Ek-Rylander, G. Andersson, J. Mol. Biol. 290 (1999) 201.
[76] S.J. Iqbal, Clin. Chim. Acta 271 (1998) 213.
[77] W.K. Lam, L.C. Lai, L.T. Yam, Clin. Chem. 24 (1978) 309.
[78] H.G. Drexler, G. Gaedicke, J. Minowada, Cancer 57 (1986) 1776.
[79] M. Nakanishi, K. Yoh, T. Miura, T. Ohasi, S.K. Rai, K. Uchida, Clin. Chem. 46 (2000) 469.
[80] T.R. Anderson, S.U. Toverud, Arch. Biochem. Biophys. 247 (1986) 131.
[81] S.H. Allen, P.R. Nuttleman, C.M. Ketcham, R.M. Roberts, J. Bone Miner. Res. 4 (1989) 47.
[82] A.J. Janckila, K. Takahashi, S.Z. Sun, L.T. Yam, J. Bone Miner. Res. 16 (2001) 788.
[83] D.W. Moss, F.D. Raymond, D.B. Wile, Crit. Rev. Clin. Lab. Sci. 32 (1995) 431.
[84] C.P. Modder, Clin. Chim. Acta 43 (1973) 205.
[85] M. Nakanishi, K. Yoh, K. Uchida, S. Maruo, A. Matsuoka, Clin. Chem. 44 (1998) 221.
[86] R. Torres, P.C. de la, A. Rapado, Calcif. Tissue Int. 49 (1991) 14.
[87] A.J. Janckila, E.M. Cardwell, L.T. Yam, C.Y. Li, Blood 85 (1995) 2839.
[88] J.D. Hoyer, C.Y. Li, L.T. Yam, C.A. Hanson, P.J. Kurtin, Am. J. Clin. Pathol. 108 (1997) 308.
[89] J.J. Stepan, J. Lachmanova, M. Strakova, V. Pacovsky, Bone Miner. 3 (1987) 177.
[90] M. Nakanishi, K. Yoh, K. Uchida, S. Maruo, S.K. Rai, A. Matsuoka, J. Bone Miner. Metab. 17 (1999) 125.
[91] J.J. Stepan, E. Silinkova-Malkova, T. Havranek, J. Formankova, M. Zichova, J. Lachmanova, M. Strakova, P. Broulik, V. Pacovsky, Clin. Chim. Acta 133 (1983) 189.
[92] K.W. Lam, C. Dannaher, S. Letchford, T. Eastlund, C.Y. Li, L.T. Yam, Clin. Chem. 30 (1984) 457.
[93] J.J. Stepan, R. Neuwirtova, V. Pacovsky, J. Formankova, E. Silinkova-Malkova, Clin. Chim. Acta 142 (1984) 203.
[94] J.J. Stepan, J. Pospichal, V. Schreiber, J. Kanka, J. Mensik, J. Presl, V. Pacovsky, Calcif. Tissue Int. 45 (1989) 273.
[95] H. Nishino, Y. Horii, T. Tanaka, T. Yamagami, T. Matsukura, S. Kagamimori, Nippon Koshu Eisei Zasshi 46 (1999) 47.
[96] F. Schiele, Y. Artur, A.Y. Floc'h, G. Siest, Clin. Chem. 34 (1988) 685.
[97] J.J. Stepan, A. Tesarova, T. Havranek, J. Jodl, J. Formankova, V. Pacovsky, Clin. Chim. Acta 151 (1985) 273.
[98] W. Withold, Eur. J. Clin. Chem. Clin. Biochem. 34 (1996) 785.
[99] D.R. Eyre, Spine 22 (1997) 17S.
[100] R. Swaminathan, Clin. Chim. Acta 313 (2001) 95.
[101] F. Paglia, S. Dionisi, S. De Geronimo, R. Rosso, E. Romagnoli, N. Raejentroph, A. Ragno, M. Celi, J. Pepe, E. D'Erasmo, S. Minisola, Clin. Chem. 47 (2001) 1314.
[102] D.D. Bikle, Am. J. Med. 103 (1997) 427.
[103] K.W. Lam, R.J. Desnick, Prog. Clin. Biol. Res. 95 (1982) 267.
[104] M. Takahashi, T. Inoue, Rinsho Byori 44 (1996) 415.
[105] R. Hannon, A. Blumsohn, K. Naylor, R. Eastell, J. Bone Miner. Res. 13 (1998) 1124.
[106] L. Scarnecchia, S. Minisola, M.T. Pacitti, V. Carnevale, E. Romagnoli, R. Rosso, G.F. Mazzuoli, Scand. J. Clin. Lab. Invest. 51 (1991) 517.
[107] K.W. Lam, L.T. Yam, Clin. Chem. 23 (1977) 89.
[108] J.M. Halleen, T.A. Hentunen, M. Karp, S.M. Kakonen, K. Pettersson, H.K. Väänänen, J. Bone Miner. Res. 13 (1998) 683.

[109] J.M. Halleen, M. Karp, S. Viloma, P. Laaksonen, J. Hellman, S.M. Kakonen, J.J. Stepan, S. Holmes, H. Väänänen, K. Pettersson, J. Bone Miner. Res. 14 (1999) 464.
[110] K. Takahashi, A.J. Janckila, S.Z. Sun, E.D. Lederer, P.C. Ray, L.T. Yam, Clin. Chim. Acta 301 (2000) 147.
[111] M. Lieberherr, J. Vreven, G. Vaes, Biochim. Biophys. Acta 293 (1973) 160.
[112] C.S. Brehme, S. Roman, J. Shaffer, R. Wolfert, J. Bone Miner. Res. 14 (1999) 311.
[113] C.T. Chu, S.V. Pizzo, Lab. Invest. 71 (1994) 792.
[114] T.R. Anderson, S.U. Toverud, Calcif. Tissue Res. 24 (1977) 187.
[115] A.J. Janckila, M.D. Latham, K.W. Lam, K.C. Chow, C.Y. Li, L.T. Yam, Clin. Biochem. 25 (1992) 437.
[116] M.E. Kraenzlin, K.H. Lau, L. Liang, T.K. Freeman, F.R. Singer, J. Stepan, D.J. Baylink, J. Clin. Endocrinol. Metab. 71 (1990) 442.
[117] J.J. Stepan, K.H. Lau, S. Mohan, M. Kraenzlin, D.J. Baylink, Biochem. Biophys. Res. Commun. 165 (1989) 1027.
[118] J.B. Vincent, M.W. Crowder, B.A. Averill, Biochemistry 30 (1991) 3025.
[119] T. Sun, K.W. Lam, L. Narukar, Clin. Chem. 27 (1981) 1742.
[120] C.K. Cheung, N.S. Panesar, C. Haines, J. Masarei, R. Swaminathan, Clin. Chem. 41 (1995) 679.
[121] J. Chen, L.T. Yam, A.J. Janckila, C.Y. Li, W.K. Lam, Clin. Chem. 25 (1979) 719.
[122] Y.R. Nakasato, A.J. Janckila, J.M. Halleen, H.K. Väänänen, S.P. Walton, L.T. Yam, Clin. Chem. 45 (1999) 2150.
[123] K.W. Lam, P. Lee, C.Y. Li, L.T. Yam, Clin. Chem. 26 (1980) 420.
[124] T. Efstratiadis, D.W. Moss, Enzyme 33 (1985) 34.
[125] M.Y. Lee, J.L. Lottsfeldt, K.L. Fevold, Blood 80 (1992) 1710.