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OSTEOCLASTIC TARTRATE-RESISTANT ACID PHOSPHATASE 5b

Diagnostic Use and Biological Significance in Bone Physiology

by

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All life is an experiment. The more experiments you make, the better.
(Ralph Waldo Emerson 1803-1882)

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Diagnostic use and biological significance in bone physiology

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ABSTRACT

The skeleton undergoes continuous turnover throughout life. In women, an increase in bone turnover is pronounced during childhood and puberty and after menopause. Bone turnover can be monitored by measuring biochemical markers of bone resorption and bone formation. Tartrate-resistant acid phosphatase (TRACP) is an enzyme secreted by osteoclasts, macrophages and dendritic cells. The secreted enzyme can be detected from the blood circulation by recently developed immunoassays. In blood circulation, the enzyme exists as two isoforms, TRACP 5a with an intact polypeptide chain and TRACP 5b in which the polypeptide chain consists of two subunits. The 5b form is predominantly secreted by osteoclasts and is thus associated with bone turnover. The secretion of TRACP 5b is not directly related to bone resorption; instead, the levels are shown to be proportional to the number of osteoclasts. Therefore, the combination of TRACP 5b and a marker reflecting bone degradation, such as C-terminal cross-linked telopeptides of type I collagen (CTX), enables a more profound analysis of the changes in bone turnover.

In this study, recombinant TRACP 5a-like protein was proteolytically processed into TRACP 5b-like two subunit form. The 5b-like form was more active both as an acid phosphatase and in producing reactive oxygen species, suggesting a possible function for TRACP 5b in osteoclastic bone resorption. Even though both TRACP 5a and 5b were detected in osteoclasts, serum TRACP 5a levels demonstrated no change in response to alendronate treatment of postmenopausal women. However, TRACP 5b levels decreased substantially, demonstrating that alendronate decreases the number of osteoclasts. This was confirmed in human osteoclast cultures, showing that alendronate decreased the number of osteoclats by inducing osteoclast apoptosis, and TRACP 5b was not secreted as an active enzyme from the apoptotic osteoclasts. In peripubertal girls, the highest levels of TRACP 5b and other bone turnover markers were observed at the time of menarche, whereas at the same time the ratio of CTX to TRACP 5b was lowest, indicating the presence of a high number of osteoclasts with decreased resorptive activity.

These results support the earlier findings that TRACP 5b is the predominant form of TRACP secreted by osteoclasts. The major source of circulating TRACP 5a remains to be established, but is most likely other cells of the macrophage-monocyte system. The results also suggest that bone turnover can be differentially affected by both osteoclast number and their resorptive activity, and provide further support for the possible clinical use of TRACP 5b as a marker of osteoclast number.

Keywords: bone turnover markers, TRACP 5a, TRACP 5b, osteoclast

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OSTEOKLASTINEN TARTRAATTI-RESISTENTTI HAPAN FOSFATAASI 5b
Diagnostinen käyttö ja biologinen merkitys luun fysiologiassa

Biolääketieteen laitos, Solubiologia ja Anatomia, Turun yliopisto
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YHTEENVETO

Luustoa muokataan jatkuvasti hajottamalla vanhaa luuta ja korvaamalla se uudisluulla. Naisilla luun aineenvaihdunta on vilkkaimmillaan murrosiässä sekä vaihdevuosien jälkeen. Luun aineenvaihduntaa voidaan arvioida mittaamalla luun hajotuksessa ja muodostuksessa vapautuvia merkkiaineita. Tartraatti-resistentti hapan fosfataasi (TRACP) on entsyymi, jota vapautuu verenkiertoon osteoklasteista, makrofaageista ja dendriittisoluista. Verenkiertoon vapautunutta TRACP:ia voidaan mitata viime aikoina kehitettyjen immunologisten määritysmenetelmien avulla. TRACP esiintyy verenkierrossa kahtena eri muotona, yhtenäisestä polypeptidiketjusta koostuvana TRACP 5a:na ja kahdesta alayksiköstä koostuvana TRACP 5b:nä, joista jälkimmäinen on peräisin osteoklasteista ja kuvaa siten luun aineenvaihduntaa. TRACP 5b:n vapautuminen ei suoraan liity luun hajotukseen, vaan on verrannollinen osteoklastien määrään. Tämän vuoksi TRACP 5b:n ja luun hajotusta kuvaavan merkkiaineen, kuten tyypin I kollageenin karboksiterminaalisen telopeptidin (CTX) yhteiskäyttö mahdollistaa tarkemman arvion luun aineenvaihdunnan muutoksista.

Tässä tutkimuksessa yhdistelmä-DNA-tekniikalla tuotettu TRACP 5a:n kaltainen proteiini muokattiin TRACP 5b:n kaltaiseksi kahdesta alayksiköstä koostuvaksi muodoksi, joka havaittiin aktiivisemmaksi sekä happamana fosfataasina että vapaiden happiradikaalien tuottajana. Tämä vahvisti aiempaa käsitystä TRACP 5b:n mahdollisesta tehtävästä luun hajotuksessa. Osteoklastien havaittiin sisältävän sekä TRACP 5a että 5b -muotoa. Vaihdevuosi-iän ohittaneilla naisilla alendronaattihoito ei kuitenkaan vaikuttanut seerumin TRACP 5a -tasoihin. Sen sijaan TRACP 5b -taso laski merkittävästi hoidon aikana, osteoklastien määrän vähenemisen seurauksena. Ihmisen osteoklastiviljelmät vahvistivat alendronaatin vähentävän osteoklastien määrää lisäämällä niiden apoptoosia, ja osoittivat, että apoptoottisista osteoklasteista ei vapaudu aktiivista TRACP 5b:tä. Murrosikäisillä tytöillä TRACP 5b sekä muut luun aineenvaihdunnan merkkiaineet olivat korkeimmillaan kuukautisten alkamisiässä, jolloin CTX:n suhde TRACP 5b:hen puolestaan oli matalin, osoittaen, että vaikka osteoklasteja on paljon, niiden luun hajotusaktiivisuus on matala.

Tulokset vahvistavat TRACP 5b:n olevan pääasiallinen osteoklasteista verenkiertoon vapautuva TRACP -muoto. Verenkierrossa esiintyvä TRACP 5a -muoto puolestaan on todennäköisimmin peräisin muista makrofaagi-monosyyttijärjestelmän soluista. Tulosten perusteella voidaan päätellä, että luun aineenvaihduntaan vaikuttavat sekä osteoklastien määrä että aktiivisuus. Tulokset myös tukevat TRACP 5b:n mahdollista kliinistä käyttöä osteoklastien määrää kuvaavana merkkiaineena.

Avainsanat: luun aineenvaihdunnan merkkiaineet, TRACP 5a, TRACP 5b, osteoklasti

CONTENTS

ABBREVIATIONS

AcP	acid phosphatase
ALP	alkaline phosphatase
Act5	immunoassay for TRACP activity
Act5a	immunoassay for TRACP 5a activity
Act5b	immunoassay for TRACP 5b activity with a selective pH
ASBI	immunoassay for TRACP 5b activity with a selective substrate
AUC	area under the curve
αvβ3	vitronectin receptor
BALP	bone-specific alkaline phosphatase
BMD	bone mineral density
BMI	body mass index
BMU	basic multicellular unit
BSA	bovine serum albumin
BSP	bone sialoprotein
CAII	carbonic anhydrase II
Cat K	cathepsin K
ClC7	chloride channel 7
CTR	calcitonin receptor
CTX	C-terminal cross-linked telopeptide of type I collagen
CV	coefficient of variation
DMP1	dentin matrix protein 1
DPD	deoxypyridinoline
DXA	dual energy X-ray absorptiometry
ELISA	enzyme-linked immunoassay
FBS	fetal bovine serum
FSD	functional secretory domain
Gla	γ-carboxyglutamic acid
HRT	hormone replacement therapy
ICTP	C-terminal telopeptide of type I collagen
IL-	interleukin-
IRMA	immunoradiometric assay
k_{cat}	catalytic constant
k_{cat}/K_m	specificity constant
kD	kilodalton
K_m	Michaelis constant
LAP	lysosomal acid phosphatase
LSC	least significant change
M-CSF	macrophage-colony stimulating factor
MiTF	microphthalamia transcription factor
mRNA	messenger ribonucleic acid
4-NPP	4-nitrophenyl phosphate
NTX	N-terminal cross-linked telopeptide of type I collagen
OC	osteocalcin, bone Gla protein
OPG	osteoprotegerin
OPN	osteopontin
PAP	purple acid phosphatase
PICP	C-terminal propeptides of type I collagen

PINP	N-terminal propeptides of type I collagen
Prot5	immunoassay for TRACP protein amount
Prot5a	immunoassay for TRACP 5a protein amount
PTH	parathyroid hormone
PYD	pyridinoline
RA	rheumatoid arthritis
RANK	receptor activator of nuclear factor kappa B
RANKL	ligand for receptor activator of nuclear factor kappa B
RIA	radioimmunoassay
ROS	reactive oxygen species
RT	room temperature
rTRACP	recombinant tartrate-resistant acid phosphatase
Runx2	osteoblast-specific transcription factor
SD	standard deviation
Sf9	*Spodoptera frugiperda 9*
TGFβ	transforming growth factor β
TRACP	tartrate-resistant acid phosphatase, formerly TRAP
V-ATPase	vacuolar-type proton adenosine triphosphatase

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals (I – IV). In addition, unpublished results are included.

I Fagerlund KM*, Ylipahkala H*, Tiitinen SL, Janckila AJ, Hamilton S, Mäentausta O, Väänänen HK and Halleen JM. (2006) Effects of proteolysis and reduction on phosphatase and ROS-generating activity of human tartrate-resistant acid phosphatase. **Arch Biochem Biophys.** 449:1-7.
* These authors contributed equally to this work.

II Rissanen JP, Ylipahkala H, Fagerlund KM, Long C, Väänänen HK, Halleen JM. (2009) Improved methods for testing antiresorptive compounds in human osteoclast cultures. **J Bone Miner Metab.** 27:105-9.

III Fagerlund KM, Janckila AJ, Ylipahkala H, Tiitinen SL, Nenonen A, Cheng S, Uusi-Rasi K, Yam LT, Väänänen HK and Halleen JM. (2008) Clinical performance of six different serum tartrate-resistant acid phosphatase assays for monitoring alendronate treatment. **Clin Lab.** 54:347-54.

IV Fagerlund KM, Lehtonen-Veromaa M, Möttönen T, Leino A, Viikari J, Väänänen HK and Halleen JM. Markers of bone turnover in peripubertal girls. **Submitted.**

1 INTRODUCTION

The skeleton undergoes continuous turnover throughout its lifetime in response to changes in mechanical loading, altered serum calcium levels and many endocrine and paracrine factors. In women, increased bone turnover is particularly pronounced during puberty and after menopause. Puberty is a period of rapid bone growth and construction of the skeleton, during which the size, shape and geometry of bone undergo a process called modeling. Modeling involves both formation and resorption, from which the former exceeds the latter and is not coupled to it temporally or spatially. After reaching the maximal bone mass, resorption and formation are tightly coupled in a process called remodeling, where old bone is continuously removed by osteoclasts and replaced by new bone formed by osteoblast in a homeostatic equilibrium. The remodeling process is essential for maintaining bone strength and repairing microdamages, as well as in regulating calcium homeostasis. In postmenopausal women, loss of ovarian sex steroids results in acceleration of bone turnover with predominance of resorption over formation. The predominance of resorption persists throughout the postmenopausal life leading to gradual loss of bone and increased risk of fractures.

Bone metabolism can be monitored by measuring biochemical markers of bone turnover from serum or urine. Bone turnover markers are products of either osteoclasts or osteoblast, and they originate from the cells themselves or from the bone matrix during resorption. These non-invasive and relatively inexpensive tools can be very helpful in the assessment of physiological bone turnover and metabolic bone diseases. Tartrate-resistant acid phosphatase (TRACP) is an enzyme expressed abundantly in osteoclasts and the use of TRACP in monitoring bone resorption has been studied since the early 1970s. It became clear that there the two different forms in the blood circulation: TRACP 5a and TRACP 5b. The role and origin of the two forms was unclear for a long time, and the earlier assays were rather unspecific and detected both forms. Earlier research conducted in Professor Väänänen's group has led to the development of a highly specific immunoassay for measuring serum TRACP 5b (Halleen et al. 2000), and subsequent evaluation of the assay has demonstrated its usefulness in the evaluation of bone turnover. TRACP 5a specific immunoassays (Chao et al. 2005a) developed recently, have suggested a completely different clinical use for this form of TRACP.

This study was undertaken to clarify the diagnostic use and biological significance of osteoclastic TRACP 5b in bone physiology. For these purposes, we produced TRACP 5a-like human recombinant protein, processed it to a TRACP 5b-like form, and studied the activity of the two forms. We used the recently developed TRACP 5a and 5b specific immunoassays in the assessment of circulating TRACP levels in postmenopausal women during alendronate treatment, and studied the effects of alendronate treatment on osteoclast number, osteoclast apoptosis and TRACP 5b secretion in human osteoclast cultures. Furthermore, we studied circulating TRACP 5b values in peripubertal girls.

2 REVIEW OF THE LITERATURE

2.1 Bone

2.1.1 Anatomy and structure

Bone is a highly specialized form of connective tissue which, together with the cartilage, makes up the skeletal system in vertebrates. It functions as a mechanical support and site of muscle attachment for locomotion, protects bone marrow and vital organs and carries a metabolic function by serving as a reserve of ions, especially calcium and phosphate, for maintenance of serum homeostasis.

Anatomically, two types of bone can be distinguished: flat bones such as the skull bones, scapula, mandible and ilium, and long bones such as tibia, femur and humerus. These two bone types are formed as a result of two distinct types of development, intramembranous and endochondral, respectively. Long bones have a cylindrical tube in the middle called the diaphysis, and wider expansions at both ends of bone called the epiphysis. The area between these two parts is called the metaphysis. In growing bone the epiphysis and metaphysis are separated by an area called the growth plate (Figure 1).

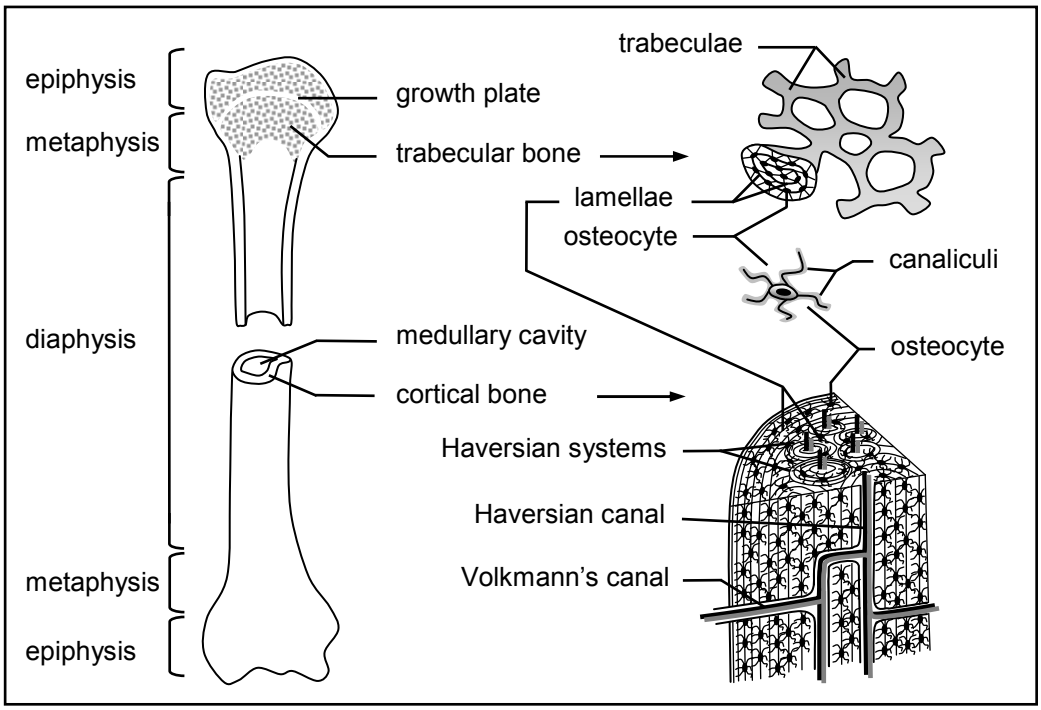

Figure 1. Structure of a long bone. Cortical bone is arranged in units called the Haversian systems (osteons). Blood vessels and nerve fibers penetrate the cortical bone through central and perforating canals called the Haversian canals and the Volkmann's canals, respectively.

Morphologically there are two forms of bone tissue: cortical (compact) and trabecular (cancellous, spongious), which comprise about 80% and 20% of the bone mass in human skeleton, respectively. The rigid and dense cortical bone is located in the outer shell of the bone, whereas the internal space consists of trabecular bone. Although the cells and matrix in these two forms of bone are similar, the structure and the function of the cortical and trabecular bone differ. In cortical bone, the matrix is highly organized and it consists of densely packed collagen fibrils, which form the concentric lamellae. Trabecular bone has a loosely organized porous matrix, and it forms an internal network of interconnecting plates and rods in the medullary cavity. The spaces enclosed by these trabeculae are filled with hematopoietic bone marrow and are in continuity with the medullary cavity of the diaphysis. Cortical bone mainly provides a mechanical and protective function, whereas trabecular bone fulfills metabolic functions (Baron 2003, Mundy et al. 2003).

2.1.2 Extracellular matrix

Bone tissue, as all connective tissues, consists of cells and extracellular matrix, from which the latter is particularly abundant. The extracellular matrix is composed of two different phases, organic and inorganic. The organic phase consists of collagen fibers and non-collagenous proteins, which provide elasticity and flexibility and also determine the structural organization of bone. The inorganic phase consists of mineral, predominantly calcium and phosphate, which provide mechanical rigidity and load bearing strength. Bone composition varies with age, state of maturation and turnover, anatomic location, diet, and health, but it is generally 50-70% mineral, 20-40% organic matrix, 5-10% water, and less than 3% lipids. The organic matrix is mainly composed of type I collagen that constitutes up to 85-90% of the total protein in the bone matrix. The remaining 10-15% in composed of noncollagenous proteins, whereas the cellular content comprises less than 1% of the bone mass (Robey and Boskey 2008).

2.1.2.1 Organic phase

The organic phase of bone matrix contains a number of proteins and proteoglycans that play a role in skeletal dynamics. Early studies focused on their role in controlling the nucleation or deposition of mineral. Presently, many of them are thought to be involved in bone remodeling, directly or indirectly, by participating in the recruitment and attachment of cells to bone (Gundberg 2003). The matrix proteins include collagen and non-collagenous proteins, from which the latter can be divided into four groups: proteoglycans, Arg-Gly-Asp (RGD) sequence containing glycoproteins, non-RGD containing glycoproteins and gamma-carboxyglutamic acid (Gla) containing proteins (Robey and Boskey 2008).

Type I collagen is the most abundant protein in bone, but it is also expressed in other connective tissues, especially in the dermis, tendons and ligaments (Myllyharju and Kivirikko 2001). Collagens, in general, can be defined as structural proteins of the extracellular matrix that contain one or more domains harboring the conformation of a collagen triple helix (van der Rest and Garrone 1991). In each of the polypeptide chains every third amino acid is glycine, and thereby the sequence of an α chain or a collagen domain in a protein can be expressed as $(Gly\text{-}X\text{-}Y)_n$, where X and Y represent

amino acids other than glycine and n varies according to the collagen type and domain. Usually X and Y represent proline and hydroxyproline, respectively. Collagen is stabilized by formation of covalent cross-links between the end of one collagen molecule and the helical portion of the adjacent collagen molecule. Despite the dominance of type I collagen in bone matrix, fibrils often contain trace amounts of type III, V and XII collagen that may be present during certain stages of bone formation and may regulate collagen fibril diameter. Type V collagen is located in the core and types III and XII on the surface of the fibril.

Proteoglycans are macromolecules that contain acidic polysaccharide chains, glycosaminoglycans, attached to a core protein. Decorin and biglycan are two heavily enriched small proteoglycans in bone matrix. Their exact physiological functions are unknown, but they are assumed to be important for the integrity of most connective tissue matrices. They both are able to bind and modulate the activity of TGF-β family members (Hildebrand et al. 1994). In transgenic mice, deletion of the biglycan gene resulted in a decrease in the development of trabecular bone, indicating that it is a positive regulator of bone formation (Xu et al. 1998). Decorin deficient mice do not have obvious skeletal defects, but they do have skin fragility that could be due to the ability of decorin to modulate collagen fibril structure and integrity (Danielson et al. 1997). Moreover, biglycan/decorin double deficient mice have more severe phenotypes in both long bones and skin (Bi et al. 2005, Corsi et al. 2002).

RGD containing glycoproteins contain a cell attachment consensus sequence, Arg-Gly-Asp, that conveys the ability of an extracellular matrix protein to bind to the integrin class of cell-surface molecules. Bone matrix contains numerous RGD containing glycoproteins, including osteopontin, bone sialoprotein, dentin matrix protein 1, matrix extracellular phosphoglycoprotein, fibronectin, thrombospondins, vitronectin and fibrillin, from which the first four belong to the SIBLING (Small Integrin-Binding LIgand, N-linked Glycoprotein) family. *Osteopontin* (OPN) is a highly phosphorylated sialoprotein that, in addition to the RGD sequence, contains a polyaspartic acid sequence and sites of Ser/Thr phosphorylation that mediate hydroxyapatite binding. As the name implies (‘bridging in bone’) it was first identified in bone matrix, but later also found to be present in several other tissues. OPN has been studied as an *in vitro* mediator of mineralization, and it seems to play an important role in the regulation of cell adhesion and motility, and bone remodeling. It may also function as a cytokine modulating inflammatory response. OPN deficient mice have no skeletal phenotype, but they are resistant to ovariectomy induced bone resorption (Yoshitake et al. 1999). *Bone sialoprotein* (BSP) is the major sialoprotein in bone. It is composed of 50% carbohydrate, from which 12% is sialic acid, and it contains stretches of polyglutamic acid. Contrary to OPN, BSP expression is restricted only to mineralized tissue and certain carcinomas that metastasize to bone. BSP deficient mice display impaired bone growth and mineralization, concomitant with reduced bone formation (Malaval et al. 2008). *Dentin matrix protein 1* (DMP1) was first identified in dentin, but afterwards found to be widely expressed in other tissues. Transgenic MC3T3-E1 cells overexpressing DMP1 show earlier onset of mineralization and produce mineralized nodules of a larger size (Narayanan et al. 2001). DMP1 deficient mice appear normal at birth, but after several days to weeks display an abnormal growth plate and delayed calcification (Ye et al. 2005). *Matrix extracellular phosphoglycoprotein* (MEPE) is highly charged and rich in potential serine phosphorylation sites. Like BSP, its expression is restricted to mineralized tissues and

certain tumors. MEPE deficient mice display increased bone mass as a result of increased osteoblast number and activity (Gowen et al. 2003). *Fibronectin* binds to fibril forming collagens, fibrin, heparin and gelatin, and it has been associated with early phases of bone formation. *Thrombospondins* are a family of five multifunctional calcium binding proteins that interact with a number of cell-surface and matrix proteins such as type I and V collagens (Grzesik and Robey 1994). *Vitronectin* is a multifunctional glycoprotein that is also present in blood. It can bind to collagens, plasminogen, plasminogen activator inhibitor, complement and heparin (Schvartz et al. 1999). *Fibrillins* are major components of microfibrils and may regulate fibril formation (Ramirez and Pereira 1999).

Non-RGD containing glycoproteins, such as alkaline phosphatase and osteonectin exhibit a broad array of functions in bone. They control cell proliferation and cell-matrix interactions, and mediate hydroxyapatite deposition and cell attachment. *Alkaline phosphatase* (ALP) is an enzyme located at the cell surface of osteoblasts. ALP can be cleaved from the surface and found within the mineralized matrix (Väänänen et al. 1987), and it probably plays an important role in the first steps of matrix formation. *Osteonectin* ('bone connector') was named after its strong affinity for collagen, hydroxyapatite and growth factors. Later it was found to be present throughout the body. It regulates cell proliferation and can stimulate angiogenesis and production of matrix metalloproteinases. Osteonectin deficient mice display decreased bone remodeling with negative bone balance, leading to profound osteopenia in older animals (Delany et al. 2000).

Gamma-carboxyglutamic acid (Gla) containing proteins are vitamin K-dependent calcium binding proteins that are post-translationally modified by γ-carboxylase. The Gla containing proteins identified in bone include bone Gla protein (osteocalcin) and matrix Gla protein. *Osteocalcin* is the most abundant non-collagenous protein in bone matrix that may function in the regulation of mineralization. Osteocalcin deficient mice exhibit increased bone mass (Ducy et al. 1996). A recent study revealed that osteocalcin also has several features of a hormone, including effects on β-cell proliferation, glucose tolerance and insulin resistance, suggesting that its role is more complex than previously thought (Lee et al. 2007). *Matrix Gla protein* may act as a negative regulator of mineralization, but contrary to osteocalcin it is found in many tissues (Luo et al. 1997).

2.1.2.2 Inorganic phase

The mineral phase consists predominantly of a calcium- and hydroxide-deficient analog of the geologic mineral, hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] with some impurities such as carbonate and magnesium. In addition, dietary cations such as magnesium and strontium can be incorporated into the bone mineral and substitute calcium. In contrast to the geologic hydroxyapatite, bone mineral crystals are substantially smaller and more soluble. This facilitates bone to act as a reservoir for calcium and phosphate and other ions. Usually, the smallest crystals are more readily dissolved and therefore lost first during remodeling. Both collagen and non-collagenous proteins influence the form of mineral deposition, and the apatite crystals are always deposited so that their longest dimension lies parallel to the axis of the collagen fibril. Initial mineral deposition begins immediately after the organic matrix

has been laid down. After the primary crystal is formed, the crystals may branch or enlarge in several dimensions. Secondary mineralization occurs as new crystal starts to form on the initial crystal. This rather slow process results in higher density of bone, and is likely to last for several months or even years (Boskey 2006). As the bone matures, the crystals become larger and more perfect. The increase in crystal dimension is due to addition of ions to the crystals and to aggregation of the crystals.

2.1.3 Cells

Osteoblasts, the bone forming cells, originate from mesenchymal stem cells that also give rise to adipocytes, chondroblasts, myoblasts and fibroblasts. Numerous hormones, growth factors and cytokines have been shown to regulate osteoblast differentiation. Key known regulators of differentiation include estrogen, parathyroid hormone (PTH), PTH-related peptide (PTHrP), insulin-like growth factors (IGFs), glucocorticoids, and vitamin D. Local growth factors influencing osteoblast maturation include bone morphogenetic proteins (BMPs), transforming growth factor β (TGFβ) and fibroblast growth factors (FGFs) (Ducy et al. 2000). The intracellular effect of these signaling molecules is mediated by the activation of specific transcription factors including Runx2 and osterix. Runx2 (previously known as Cbfa1) has been identified as the master regulator controlling osteoblast commitment and differentiation. It is the earliest and most specific marker of osteoblastogenesis. Runx2 deficient mice have a cartilaginous skeleton without any osteoblasts (Komori et al. 1997). However, Runx2 alone is not sufficient to induce osteoblastogenesis. Osterix, a downstream factor for Runx2, is a zinc finger containing transcription factor required in the later stages of osteoblast differentiation. Osterix deficient mice show absence of osteoblasts and defective bone formation (Nakashima et al. 2002).

The process of osteoblast differentiation can be divided into three stages: proliferation, extracellular matrix production and maturation, and mineralization. During the first stage, the osteoprogenitor cells proliferate and begin expressing type I collagen, and transiently also osteopontin. During the second stage, osteoblasts form the bone matrix by producing type I collagen, bone sialoprotein and alkaline phosphatase. In the third, mineralization stage, the cells begin to express osteocalcin, osteopontin and collagenase. The active, matrix producing osteoblasts have a large nucleus, enlarged Golgi and the extensive endoplasmic reticulum typical of a cell engaged in secretion. The active osteoblasts are also highly enriched in ALP and secrete type I collagen and other bone matrix proteins (Lian et al. 2003). When bone formation is complete, some osteoblasts undergo apoptosis whereas others become quiescent. Flat, elongated cells observed on quiescent bone surface are called bone lining cells. The origin of these cells is still unclear, but they can differentiate into osteogenic cells representing a possible subpopulation of osteoblasts, which may have a role in the regulation of bone remodeling (Everts et al. 2002).

Osteocytes are the most abundant cells in bone representing the final differentiation stage of osteoblasts embedded within the mineralized bone matrix. They are non-proliferative, spindle-shaped cells that occupy a space called lacuna within the matrix. Nascent osteocytes produce a high amount of osteocalcin, osteonectin and osteopontin, but appear to be negative for ALP. Osteocytes within the mineralized matrix are in direct communication with each other and with surface osteoblasts and lining cells

through numerous cellular processes that pass through the matrix via small canals. These canals form a thin canalicular network, which permeates the whole bone matrix (Figure 1). The cellular processes within the canaliculi are connected by gap junctions that allow the cells to communicate with each other. Moreover, the canalicular network is essential for osteocyte survival, because it is the route of metabolic traffic and exchange (Lian et al. 2003).

It has been suggested that 10-20% of osteoblasts differentiate into osteocytes (Aubin and Turksen 1996). The decision to become an osteocyte involves many significant changes, including reduced susceptibility to apoptotic death, permanent removal from the cell cycle, and production of dendritic processes. The transformation from motile osteoblast to entrapped osteocyte takes about three days, during which the cell produces a volume of extracellular matrix three times its own cellular volume (Palumbo 1986). In healthy bone, the mature osteocytes have the potential to live for decades. However, empty lacunae are observed in aging bone, suggesting that the cells may undergo apoptosis (Noble et al. 1997). The properties and functions of osteocytes are still poorly understood. However, several theories have been proposed. They have been suggested to act as mechanosensors for stress and the loading of bone. The transmission of mechanical signals to the osteocyte cytoskeleton via cell surface receptors can occur directly through the solid matrix structure or indirectly via fluid pressure and shear stress imparted by fluid flow. Osteocytes are involved in the control of bone remodeling since osteocyte death is eventually followed by matrix resorption. They also produce signaling molecules such as sclerostin, DMP1 and nitric oxide (NO), which modulate the activity of osteoblasts. They have also been suggested to regulate mineral homeostasis by a process called osteocytic osteolysis (reviewed by Noble 2008). Recent studies have demonstrated osteocytes as the main source of fibroblast growth factor 23 (FGF23) that works as a signal from bone to kidney, inhibiting phosphate reabsorption and $1,25(OH)_2D$ production, and participating as the principle phosphaturic factor in a bone-kidney axis (Liu et al. 2006, Sitara et al. 2004). These findings establish a novel concept that bone also works as a hormone-producing organ.

Osteoclasts, the multinucleated giant cells specialized in bone resorption, originate from hematopoietic stem cells. They are members of the monocyte/macrophage lineage and are formed via multiple cellular fusions from their mononuclear precursors. The same precursors also give rise to different tissue macrophages and dendritic cells (Takahashi et al. 2008). Individual transcription factors for osteoclastogenesis include PU.1, microphthalmia (MiTF), NFκB and c-Fos. PU.1 and MiTF have been demonstrated to take part in the commitment of hematopoietic stem cells into the myeloid lineage and into the early differentiation of macrophages, whereas c-Fos is essential for osteoclastogenesis after lineage separation with macrophages (Baron 2003, Takahashi et al. 2008). Osteoclastogenesis is mediated by cell-cell contact between osteoblast/stromal cells and osteoclast precursors and modulated by various cytokines and hormones. Several cytokines, most importantly macrophage colony stimulating factor (M-CSF), a ligand for receptor activator of nuclear factor kappa B (RANKL) and osteoprotegerin (OPG) are known to affect osteoclastogenesis. These three essential factors are produced by osteoblasts/stromal cells. M-CSF induces the proliferation of osteoclast precursor cells and supports their survival and expression of receptor activator of nuclear factor kappa B (RANK). The later differentiation of osteoclasts is dependent on the presence of RANKL on the surface of osteoblasts/stromal cells. OPG, a soluble “decoy” receptor that competes with RANK

for RANKL, is an effective inhibitor of osteoclast formation. These components are expressed at different stages of osteoblast differentiation, and the ratio of OPG and RANKL is critical for osteoclastogenesis. The crucial role of these cytokines in osteoclastogenesis has been demonstrated in RANK (Kong et al. 1999), RANKL (Dougall et al. 1999) and M-CSF (Yoshida et al. 1990) deficient mice that fail to produce osteoclasts. In addition to these, numerous other cytokines and hormones, including estrogen, PTH, 1,25-dihydroxyvitamin D, thyroid hormones, glucocorticoids, TNFα, IL-1, IL-11 and PGE-2, regulate osteoclastogenesis by affecting the RANK-RANKL-OPG system. Of these, estrogen has a broad range of effects in regulating osteoclastogenesis and bone resorption. It inhibits PTH-induced osteoclastogenesis and osteoclast attachment to bone. Further, several cytokines that prolong osteoclast survival, including IL-1, IL-6, TNFα, M-CSF and RANKL, are negatively regulated by estrogen, whereas the synthesis of antiresorptive factors such as OPG and TGFβ is stimulated. In addition to RANK-RANKL-OPG, other regulatory mechanisms of osteoclastogenesis exist. One example of such regulatory mechanism is calcitonin, a polypeptide hormone secreted by thyroid C-cells in response to hypercalcemia, that binds to the calcitonin receptor (CTR) expressed by mature osteoclasts and rapidly terminates resorption activity (Lian and Stein 2006).

Bone resorption is a complex and energy demanding process, which is enabled by the special characteristics of the osteoclasts. The cells have an exceptionally high number of mitochondria, abundant Golgi complexes around the nuclei and cytoplasm containing numerous vacuoles that are usually loaded with lysosomal enzymes. The cytoplasm is highly organized and interconnects the nuclei with other cellular organelles and compartments. The two most dramatic features of the cytoskeleton are its ruffled membrane and actin ring which are both formed when the cell contacts bone (Lakkakorpi et al. 1989). The other special characteristics of the mature, resorbing osteoclast include expression of c-Src, vitronectin receptor (αvβ3), vacuolar ATPase (V-ATPase), chloride channel 7 (ClC7), carbonic anhydrase II (CAII), cathepsin K (Cat K), TRACP and CTR (Figure 2). c-Src and αvβ3 are involved in the attachment and polarization of the cells, whereas V-ATPase, ClC7, CAII and Cat K are essential for resorption activity. V-ATPase mediates proton transport and acidification of the resorption lacuna, enabling dissolution of the mineral crystals. The counter anions, Cl^-, are transported to the lacuna via the chloride specific ion channel, ClC7. An efficient proton supply is ensured by a high content of cytosolic CAII, which facilitates the hydration of CO_2. The H_2CO_3 formed then spontaneously dissociates to protons (H^+) and bicarbonate (HCO_3^-), from which the protons are used by V-ATPase and the bicarbonate is extruded from the cells via an anion exchanger (reviewed by Suda et al. 1992, Teitelbaum 2000). Dissolution of mineral is followed by secretion of proteases, mainly Cat K, which is able to degrade the remaining insoluble type I collagen at the acidic pH. In addition to Cat K, other osteoclastic proteases such as matrix metalloproteinases may contribute to the degradation of the organic matrix (Everts et al. 2006). TRACP expression is dramatically increased during osteoclast differentiation, and it is also used as a cellular marker of osteoclasts. However, TRACP is also expressed in other members of the monocyte/macrophage lineage, including activated macrophages and dendritic cells. TRACP functions as an acid phosphatase and it is also capable of generating reactive oxygen species (ROS). The ROS generating activity of TRACP has been shown to facilitate collagen degradation and it may therefore have a role in the final degradation of the resorption products (Väänänen and Zhao 2008).

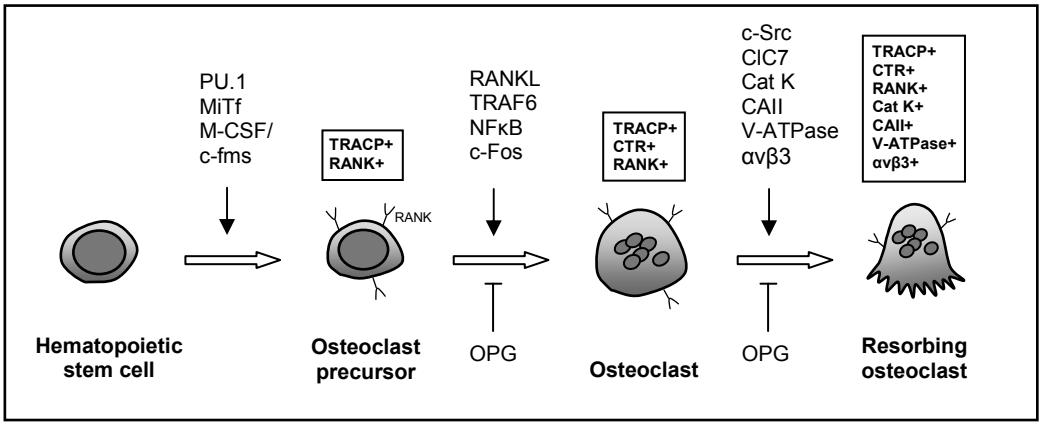

Figure 2. Differentiation of osteoclasts. PU.1, a myeloid- and B-cell specific transcription factor; **MiTF**, microphthalmia transcription factor; **M-CSF**, macrophage colony stimulating factor; **c-fms**, receptor for M-CSF; **RANK(L)**, receptor for activator of nuclear factor kappa B (ligand); **OPG**, osteoprotegerin; **TRAF6**, tumor necrosis factor receptor associated factor 6; **NFκB**, nuclear factor kappa B; **ClC7**, chloride channel 7; **Cat K**, cathepsin K; **CAII**, carbonic anhydrase II; **V-ATPase**, vacuolar-type proton adenosine triphosphatase; **αvβ3**, vitronectin receptor ; **TRACP**, tartrate-resistant acid phosphatase; **CTR**, calcitonin receptor.

The osteoclastic resorption cycle is a multistep process that begins with recruitment and differentiation of osteoclast progenitor cells that then fuse to form a multinuclear mature osteoclast (Lakkakorpi and Väänänen 1996). Mature osteoclasts attach to the bone surface via a distinct integrin receptor that binds to a variety of extracellular RGD containing proteins, including vitronectin, collagen, osteopontin and bone sialoprotein. The attached cells polarize and form three distinct membrane domains: the basolateral membrane, the sealing zone and the ruffled border. The sealing zone attaches the plasma membrane firmly to the bone surface and encloses an area called the resorption lacuna. The actual resorption occurs in the resorption lacuna via the ruffled border, which displays the characteristics of the late endosomal membrane. A fourth membrane domain, called the functional secretory domain (FSD), appears on the top of the cell when matrix degradation is initiated (reviewed by Väänänen et al. 2000). The organic and inorganic degradation products are endocytosed to the resorbing cell, transported through the cell in vesicles, and secreted into the extracellular matrix via the FSD (Nesbitt and Horton 1997, Salo et al. 1997). Thus, osteoclasts are able to continuously remove a large amount of degradation products without loosening the tight attachment to bone. The transcytotic route also provides a possibility for further degradation of the resorption products. When the resorption is complete the cells either become apoptotic or start another resorption cycle (Väänänen and Zhao 2008).

2.2 Bone turnover

2.2.1 Growth and modeling

2.2.1.1 Bone accrual

Bone mass increases dramatically in childhood and adolescence, being approximately 70-95 g at birth and increasing to 2.4-3.3 kg in young women and men, respectively. This gain is achieved through longitudinal and appositional growth and modeling. In humans, the major bone gain occurs during the first two decades of life, which includes two growth spurts: the first at the age of one to three years and the second during puberty. During the longitudinal bone growth the cortex in the diaphysis is resorbed inside and reformed outside and the growth plate moves upward. Growth in the diameter of the shaft is the result of a deposition of new membranous bone beneath the periosteum. In this case, resorption does not immediately precede formation (Baron 2003). Modeling is the process by which the skeleton is constructed. It changes the size, shape and geometry of bone in childhood, adolescence and also in adulthood in response to changes in loading patterns. The process of bone modeling involves both formation and resorption, from which the former exceeds the latter and is not coupled to it temporally or spatially.

During growth, the skeleton responds to the changing environment. The skeleton's adaptation to functional demands was already discovered in 1892, and it has thereafter been referred to as Wolff's law (Wolff 1892). According to Wolff's law, the architecture of bone is determined by mechanical stress and bone can adapt its mass and structure to obtain higher efficiency and load bearing. Modeling can determine and increase bone mass and strength, but seldom if ever reduces them (Frost 1997). Frost described the Utah paradigm of skeletal physiology, which elaborated Wolff's law and placed it within the context of the relationship between bone and muscle development (Frost 1998). He suggested that when strain reaches a minimum magnitude, the modeling phase is switched on, and he called this strain the minimum effective strain for modeling (MESm). MESm was suggested to be approximately 1000 microstrain units (μE), which in compression would shorten a bone by 0.1% of its original length. Strains below this would not induce modeling. In rapidly growing children the steadily increasing body weight and muscle strength could induce a lag between the bone's structural adaptation and the mechanical need, and as a result, the strain would exceed the modeling threshold (Frost 1997). Growth in muscle strength and body weight tend to plateau around skeletal maturity, and thereafter between 20 to 30 years of age the bone mass, strength and architecture catches up the mechanical need, and the mechanically controlled modeling ceases. However, in the mature adult skeleton, modeling may also occur in response to altered biomechanical stress, which can be induced e.g. by vigorous exercise, but the capacity of the skeleton to respond in this way decreases with increasing age. Modeling is also involved in the fracture healing process, although fracture healing mainly occurs via endochondral bone formation.

Other factors influencing skeletal growth and modeling include genetic factors, sex, ethnicity, environment and hormonal status. Data obtained from mother-daughter pairs, sib pairs and twin studies have estimated the heritability of bone mass to account for 60-80% of its variance (Christian et al. 1989, Pocock et al. 1987, Seeman et al. 1989). The extent of the genetic effect varies with age and skeletal site, being higher in the young than in the elderly. Sex steroids, predominantly estrogens and androgens, have

major effects on skeletal growth and maturation. They are responsible for the sexual dimorphism of the skeleton, initiation of pubertal increase of growth velocity, and closure of the epiphyseal growth plates, which results in cessation of longitudinal growth of the long bones. Data obtained from prepubertal boys and girls shows no difference in bone mineral density (BMD), indicating that sex affects the bone mass only after puberty (Nelson et al. 1997, Nguyen et al. 2001). Ethnic comparisons in the U.S. population suggests a higher bone mass among African-Americans compared with Caucasians, which seems to be present throughout growth (Bell et al. 1991, Nelson et al. 1997). The type of physical activity and intensity, dietary calcium intake, hormonal status and vitamin D levels also affect bone growth (Ducher et al. 2008, Winzenberg and Jones 2008). Vitamin D status is particularly important to notice at high latitudes such as in Finland, where a substantial proportion of adolescents are vitamin D deficient during the winter (Lehtonen-Veromaa et al. 1999).

2.2.1.2 Peak bone mass

Peak bone mass has been defined as the amount of bony tissue present at the end of skeletal maturation (Bonjour et al. 1994). It is thought to be an important determinant of bone mass in later life but the age when peak bone mass is obtained is still unclear. Earlier studies reported the peak value to be achieved during the 3rd and 4th decades of life (Ott 1990, Recker et al. 1992). However, it has been more generally accepted that the majority of bone mass accumulation is achieved before the end of the 2nd decade of life and followed by a period of consolidation (Bonjour et al. 1991, Haapasalo et al. 1996, Henry et al. 2004, Lin et al. 2003).

In prepubertal children there is a close relationship between bone mass and body height, but this becomes less evident during puberty. In girls, the rate of increase in bone mass decreases rapidly after the menarche, whereas in boys the gains in bone mass persist up to 17 years of age and are closely related to the pubertal stage and androgen status. By the age of 20 in both sexes the vast majority of peak bone mass has already been achieved, but some increases in bone mass during the third decade of life have been demonstrated (Bonjour et al. 1991, Henry et al. 2004, Parsons et al. 1996). In addition, the rate of bone growth has been shown to be region specific and the peak bone mass is reached earlier e.g. in the femoral neck than in lumbar spine (Henry et al. 2004, Lin et al. 2003). The achievement of high peak bone mass early in life can play a critical role against postmenopausal or age related bone loss. However, two possibilities may account for the susceptibility to fractures in later life: either increased rate of bone loss, or lower peak bone mass in young adulthood and thereafter bone loss at the same rate and length of time as those without fractures (Schönau 2004).

2.2.2 Remodeling

Bone is a dynamic tissue that is constantly resorbed and formed in response to changes in mechanical loading, serum calcium levels, and many endocrine and paracrine factors. In a homeostatic equilibrium resorption and formation are balanced so that old bone is continuously replaced by new tissue. This remodeling process is essential for maintaining bone strength and repairing microdamage, and it also has a role in

regulating calcium homeostasis. Remodeling occurs in both trabecular bone surfaces and in the Haversian system of the cortical bone, but the rate of the process differs. At any given time, approximately 10-15% of the bone surface is undergoing remodeling, the remaining surface being relatively quiescent (Kanis et al. 1995).

The remodeling process is accomplished by assembly of osteoclasts and osteoblasts into discrete temporary anatomic structures called basic multicellular units (BMUs) (Frost 1990). It has been estimated that the human skeleton contains approximately 1-2 x 10^6 such units (Riggs and Parfitt 2005). The organization of the BMUs in cortical and trabecular bone differs. In cortical bone the BMU forms a cylindrical canal of approximately 2 000 μm long and 150-200 μm wide and gradually burrows through the bone with a speed of 20-40 μm/day. During a remodeling cycle approximately ten osteoclasts dig a circular tunnel in the dominant loading direction, following an entry of several thousand osteoblasts that fill the tunnel (Parfitt 1994, Petrtyl et al. 1996). In trabecular bone osteoclasts travel across the trabecular surface with a speed of approximately 25 μm/day, digging pits of 40-60 μm in depth. The remodeling cycle of each BMU includes various sequential events (Figure 3).

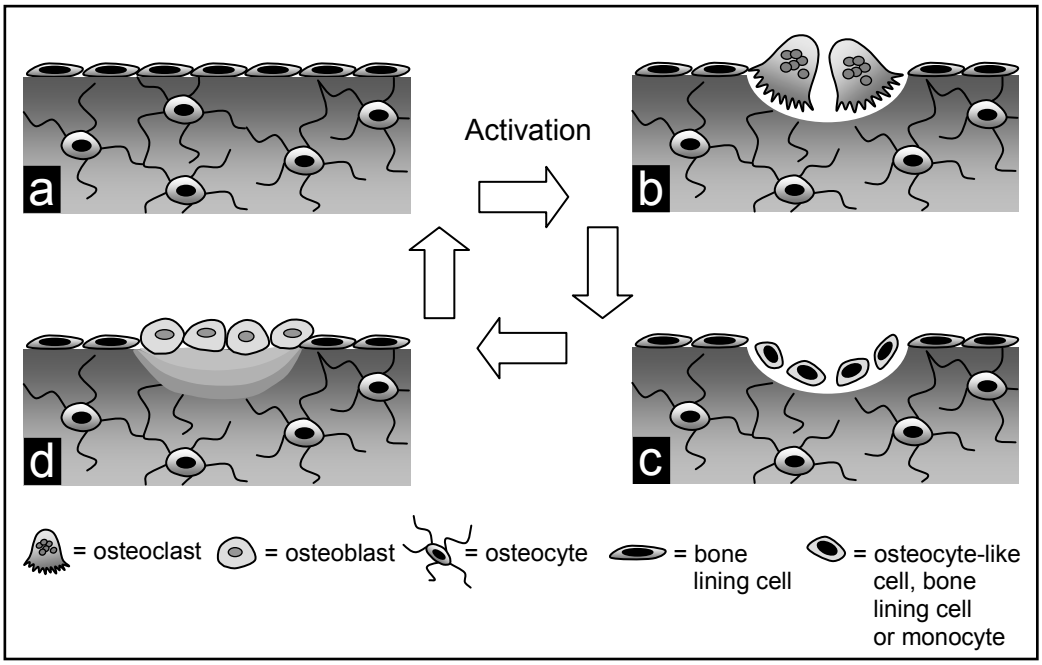

Figure 3. Bone remodeling cycle. Remodeling consists of a sequence of tightly regulated events: a) resting phase, b) resorption phase, c) reversal phase, d) formation and mineralization phase.

During the *resting phase,* no resorption or formation takes place. During *activation* partially differentiated mononuclear preosteoclasts migrate to the future resorption site where they fuse to multinucleated osteoclasts. The bone lining cells retract and the mature osteoclasts occupy space on the bone surface. The osteoclast formation may be initiated by a range of local factors released from nearby osteoblast lineage cells, which

include osteoblasts, preosteoblasts, lining cells and osteocytes. It has been suggested that osteocytes inhibit the bone lining cell mediated BMU origination and activation of osteoclasts (Gu et al. 2005, Martin 2000). Stimulating factors can be derived from the cells within the marrow, including the immune cells. The *resorption phase* starts after the osteoclasts have attached to bone, polarized, and formed the ruffled border. The detailed resorption cycle of the osteoclast has been described earlier (see 2.1.3). The resorption phase requires only about 2–4 weeks to complete and is terminated when the osteoclasts undergo apoptosis or return to the non-resorbing stage. The *reversal phase* intermediates the resorption and formation. During this phase osteoblast-like cells (Mulari et al. 2004), bone lining cells (Everts et al. 2002), or monocytes (Tran Van et al. 1982) appear on the resorbed bone surface and prepare it for osteoblasts. They may provide signals for osteoblast differentiation and migration, and have been reported to be capable of functioning as matrix degrading cells and of finalizing the resorption phase.

Exposed matrix proteins in the resorption pit, such as type I collagen and osteocalcin, have been suggested to function as chemoattractants for osteoblasts (Mundy et al. 1982). These and some growth factors induce osteoblast precursors to proliferate and differentiate at the remodeling site. During the *formation and mineralization phase* osteoblasts lay down new bone until the resorbed area is replaced with new bone. The old and new bone matrices are linked to each other by a cement line, a specialized matrix rich in osteopontin and other bone phosphoproteins and proteoglycans (McKee and Nanci 1996). When the resorption cavity has been filled, the bone formation ceases and osteoblasts remaining inside the new bone become osteocytes. The newly synthesized organic matrix, which has not yet been mineralized, called osteoid, is observed during a time lag between matrix formation and its mineralization. The mineralization of the osteoid proceeds was described earlier (see 2.1.3). The remaining osteoblasts either undergo apoptosis or become quiescent. The bone surface is covered with flattened lining cells and a prolonged *resting phase* begins and continues until a new remodeling cycle is initiated.

The phases of the remodelling cycle have different lengths. The resorption phase requires only about 2–4 weeks to complete, the reversal phase may last up to 4-5 weeks, and the formation phase can continue for several months (reviewed by Hadjidakis and Androulakis 2006). The most important regulators of the remodeling process include both systemic and local factors. The systemic factors include PTH, calcitriol, calcitonin, glucocorticoids, thyroid hormones and estrogens. Many of these and a number of other cytokines and hormones exert their effects on osteoclastogenesis by regulating the local OPG-RANK-RANKL system.

2.2.3 Bone loss and osteoporosis

After reaching peak bone mass, the amount of bone starts to decrease slowly. Between the ages of 20 to 40 years, integrity and bone mass are relatively stable. Thereafter, bone loss occurs due to hormonal changes and declines in physical activity and muscle mass. In women, there is an accelerated rate of bone loss at the time of menopause, with a probable duration of five to ten years. Estrogen deficiency has long been recognized as a major cause of bone loss in the first decade after menopause. In men, relatively little data is available, but bone loss is believed to accelerate after the age of

70 years (Szulc and Delmas 2001). The mechanisms of bone loss in older men are unclear, but elevated PTH and diminished testosterone levels are postulated as contributing factors. Women have been estimated to lose half of their bone mass by the age of 80 years, whereas the corresponding reduction in males is 25-30% (Väänänen 1996). Age-induced bone loss results from the negative balance of osteoblastic activity relative to osteoclastic activity, but it may also be contributed to by reductions in both intestinal and renal tubular absorption of calcium. Because bone loss persists throughout life, it results in high risk of fractures in subjects whose peak bone mass was in the lower part of the normal range.

Osteoporosis is a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue with a consequent increase in bone fragility and susceptibility to fracture (Anonymous 1993). It is often called a silent disease because bone loss occurs without symptoms, and it is clinically recognized by the occurrence of low-trauma fractures usually located in the hip, spine and distal radius. The occurrence of one or multiple fractures significantly affects morbidity, mortality and economic costs. The overall lifetime risk for any type of osteoporotic fracture is estimated to be approximately 40-50% for women and 13-22% for men at the age of 50 years (Johnell and Kanis 2005).

In 1994, the World Health Organization published diagnostic criteria for osteoporosis (WHO 1994). The diagnosis is based on the assessment of BMD by dual-energy x-ray absorptiometry (DXA). The original criteria for the diagnosis of osteoporosis included measurements at femoral neck, lumbar spine and forearm. Later, femoral neck was recommended as the site for diagnosis and as a reference data because of its extensive validation through meta-analyses that have carefully characterized the gradient of fracture risk at this site (Kanis et al. 2008). The distribution of BMD in young healthy adults is approximately normally distributed, irrespective of the measurement technique used. Therefore, the BMD values in individuals may be expressed in relation to a reference population in standard deviation (SD) units. The Finnish reference database concists of healthy Finnish women aged 20-29 years (Kröger et al. 1992). SDs calculated in relation to the mean of young healthy population are referred to as the T-score. Four general diagnostic categories are proposed for men aged 50 years or more and postmenopausal women using measurements of DXA at the femoral neck: 1) Normal; a value for BMD that is higher than 1 SD below the young adult female reference mean, 2) Low bone mass (osteopenia); a value for BMD more than 1 SD below the young female adult mean, but less than 2.5 SD below the value, 3) Osteoporosis; a value for BMD 2.5 SD or more below the young female adult mean, and 4) Severe osteoporosis; a value for BMD 2.5 SD or more below the young female adult mean in the presence of one or more fragility fractures. In the young healthy female population, approximately 15% have low bone mass or osteopenia, whereas only approximately 0.5% fall into the osteoporotic range (Figure 4). The proportion of women affected by osteoporosis increases greatly with age, but the normal distribution of BMD remains (Kanis 2002). However, there are some limitations in the application of DXA for the diagnosis of osteoporosis. The DXA image is two dimensional and therefore provides an areal BMD rather than a true volumetric BMD. As a consequence, the computation of BMD is sensitive to changes in bone size. It should also be noticed that BMD is an index of bone mass only when bone is fully mineralized. The presence of osteomalacia, often caused by poor nutrition in the elderly, results in

underestimation of the bone mass. Osteoarthritis at the spine or hip, also common in the elderly, contributes to heterogeneity in BMD measurements but not necessarily to bone strength. In addition, changes of fat distribution can cause alterations in DXA measurement without any real change in the skeleton (Tothill et al. 1997). Moreover, it is often argued that the definition of osteoporosis focuses too much on bone mass, rather than on bone strength.

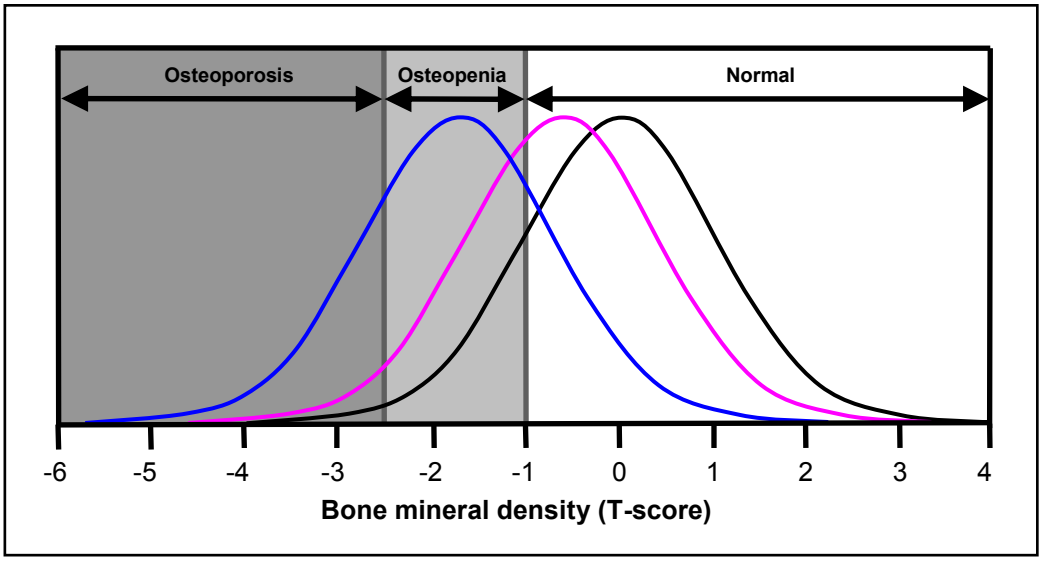

Figure 4. Distribution of bone mineral density in women aged 30-40 years (black line), 50-59 years (purple line) and 70-79 years (blue line) (modified from Kanis 2002).

Several risk factors can predispose the population to osteoporotic fractures. Over the past years several meta-analyses have been performed to identify clinical risk factors for fractures and to determine their dependence upon age, sex and BMD (De Laet et al. 2005, Johnell et al. 2005, Kanis et al. 2004a, Kanis et al. 2004b, Kanis et al. 2004c, Kanis et al. 2005a, Kanis et al. 2005b). In addition to BMD, the risk factors include low body mass index (BMI), advanced age, female gender, poor nutrition, physical inactivity, cigarette smoking, alcohol consumption, sex hormone deficiency and genetic factors. Low BMI was shown to confer a risk for all fractures, which is largely independent of age but dependent of BMD. The contribution to fracture risk was much more marked at low BMI values than at values above the median (De Laet et al. 2005). Calcium and vitamin D deficiency are common causes of increased bone resorption in the elderly, and they are caused by a number of factors including dietary changes, malabsorption and lack of sunlight exposure. A multiplicity of environmental factors makes the determination of fracture heritability complicated, although genetic components have been suggested to play a role. The combination of clinical risk factors and BMD provide higher specificity and sensitivity than either alone (Kanis et al. 2007), and assessment of fracture risk should should not be based solely on BMD measurement anymore. To improve identification of patients at high risk of fracture, WHO has developed a Fracture Risk

Assessment Tool (FRAX®, www.shef.ac.uk/FRAX/) that can be used for prediction of ten year risk of osteoporotic fractures. This online tool is based on individual patient models that integrate the risks associated with clinical risk factors as well as femoral neck BMD. In Finland, a similar tool called Mikkeli Osteoporosis Index (MOI) is widely used (Waris et al. 2005).

The aim of prevention and treatment of osteoporosis is to prevent the occurrence of future fractures. Non-pharmacological interventions include calcium and vitamin D supplementation, exercise and prevention of falls. Pharmacologic therapy for postmenopausal osteoporosis includes hormone replacement therapy (HRT), bisphosphonates, calcitonin, teriparatide and strontium ranelate. Estrogen ± progestin was the earliest recognized antiresorptive therapy that countered the bone turnover acceleration that occurs after menopause. However, the use of estrogen for preventing osteoporosis is declining because of the increased risk of cancer and cardio-vascular diseases (Kleerekoper 2008). Raloxifene, a selective estrogen receptor modulator (SERM), has an agonist effect on bone and antagonist effects on breast and endometrium. Several other SERMs are also being developed (Lindsay 2008). Bisphosphonates, the synthetic analogues of inorganic pyrophosphate, are the most potent inhibitors of bone resorption. Because of their efficacy, safety and ease of administration, they are generally accepted as the first-line therapy for osteoporosis. As a result of their strong affinity for hydroxyapatite, the compounds are incorporated in bone, and released and taken up by osteoclasts in the acidic environment of bone resorption lacunae. Currently alendronate, ibandronate, risendronate and zolendronate are widely approved for the treatment of osteoporosis, whereas clodronate, etidronate and pamidronate are available in some countries. Of these, the non-nitrogen containing bisphosphonates (clodronate and etidronate) incorporate into ATP and generate metabolites that induce osteoclast apoptosis (Selander et al. 1996). Nitrogen-containing bisphosphonates (alendronate, ibandronate, risendronate, zolendronate and pamidronate) inhibit farnesyl pyrophosphate synthase, an enzyme of the mevalonate biosynthetic pathway, and thereby decrease the prenylation of small GTPases (Rogers et al. 1999). Subsequent changes in cytoskeletal integrity leads to inactivation and potentially apoptosis of the cells. Calcitonin, a peptide hormone that binds to its receptor on osteoclasts and inhibits bone resorption, has been used as a therapeutic agent for a long time. It is indicated in several countries for the treatment of postmenopausal osteoporosis at least five years after menopause, and its use is often limited to patients who fail to respond or cannot tolerate bisphosphonate therapy, or with less severe forms of osteoporosis. Calcitonin has also been reported to reduce pain associated with vertebral fractures (Adami 2008). More recently, two compounds that directly stimulate bone formation, teriparatide, the recombinant 1-34 fragment of PTH, and strontium ranelate, have been approved for treatment of osteoporosis. Data obtained from clinical trials indicates greater improvements in bone mass with the anabolic drugs than with antiresorptives. However, antifracture effectiveness has not been fully compared with antiresorptives, and in general, use of the anabolic agents is reserved for patients with severe osteoporosis (Kleerekoper 2008). The use of teriparatide is also limited by its subcutaneous daily injections, costs, and by lack of long-term safety.

New insights of the metabolic pathways covering the osteoblast/osteoclast formation and function have identified specific points of intervention and are leading to the development of the next generation of therapies for osteoporosis. Potential antiresorptive agents include inhibitors of RANKL (denosumab), Cat K (balicatib, relacatib and CRA-013783), V^+ATPase, ClC7, c-Src (AZD0530), and disintegrins that bind with high affinity to αvβ3. Of these, RANKL inhibitors act predominantly by inhibiting osteoclast formation, whereas the other agents act by inhibiting osteoclast function. Inhibitors of Cat K, V^+ATPase, ClC7 and c-Src constitute a potentially interesting new class of antiresorptive compounds that could inhibit bone resorption without decreasing the number of osteoclasts. Subsequent uncoupling of bone formation from bone resorption might result in inhibition of bone resorption without concomitant inhibition of bone formation. In the control of bone formation, particular interest has been focused in the possibility of modulating the activity of components of the Wnt canonical signaling pathway. Possibly suitable targets include extracellular agonists and the points of interaction of agonists, especially the secreted frizzled-related proteins, dickkopf proteins and sclerostin, as well as intracellular glycogen synthase kinase -3β. However, studies with these molecules are still either in the preclinical or early investigational stages without data on fracture prediction efficacy (Ng and Martin 2008).

2.3 Biochemical markers of bone turnover

The rate of bone formation and resorption can be evaluated either by measuring predominantly osteoblastic or osteoclastic enzyme activities or by assaying bone matrix components released in the blood circulation and excreted in the urine. Over the past 25 years, research has generated a number of novel and specific biochemical markers that have enriched the spectrum of analytes in the assessment of skeletal pathologies. They are non-invasive, relatively inexpensive, and when applied and interpreted correctly, helpful tools in the diagnostic and therapeutic assessment of metabolic bone diseases (Cremers et al. 2008). Traditionally, the markers have been separated into markers of formation and markers of resorption. However, because the markers either originate from the cells itself or from the bone matrix during resorption (Figure 5), they can also be classified as markers that reflect (1) bone resorption, (2) osteoclast number, (3) bone formation, and (4) osteoblast differentiation (Sørensen et al. 2007) (Table 1). However, the distinction of a marker is not always that clear. Some marker components, e.g. certain osteocalcin fragments, reflect both formation and resorption. The events of formation and resorption are usually coupled and therefore any marker usually reflects the overall rate of bone turnover. In the following two sections, the most sensitive and new potential markers of bone turnover will be discussed. One resorption marker, hydroxyproline, with a long history of use has been excluded from the discussion because of its lack of bone specificity. Consequently, its use has been largely replaced by more specific and simple techniques.

Table 1. Markers of bone a) formation and b) resorption (modified from Seibel 2006 and Sørensen et al. 2007).

a) Marker	Origin	Process	Specimen	Methods
PINP	bone, soft tissue, skin	bone formation	serum	RIA, ELISA
PICP	bone, soft tissue, skin	bone formation	serum	RIA, ELISA
BALP	bone	osteoblast differentiation	serum	IRMA, EIA, precipitation
OC	bone, platelets	bone formation	serum	RIA, IRMA, ELISA

Abbreviations: PINP, N-terminal propeptide of type I collagen; PICP, C-terminal propeptide of type I collagen; BALP, bone specific alkaline phosphatase; OC, osteocalcin; IRMA, immunoradiometric assay; EIA, enzyme immunoassay; RIA, radioimmunoassay; ELISA, enzyme-linked immunosorbent assay.

b) Marker	Origin	Process	Specimen	Methods
PYD	bone, cartilage, tendon, vessels	bone resorption	urine, serum	HPLC, ELISA
DPD	bone, dentin	bone resorption	urine, serum	HPLC, ELISA
Gal-Hyl	bone, skin	bone resorption	urine, serum	HPLC, ELISA
NTX	tissues containing type I collagen	bone resorption	urine, serum	ELISA, CLIA, RIA
CTX	tissues containing type I collagen	bone resorption	urine (α/β), serum (β)	ELISA, RIA
ICTP	bone, skin	pathological bone resorption	serum	RIA
TRACP 5b	osteoclasts	osteoclast number	plasma, serum	colorimetry, RIA, ELISA
Cat K	primarily osteoclasts	osteoclast number (?)	plasma, serum	ELISA
OC-f	bone	bone resorption	urine	ELISA
BSP	bone, dentin, hypertrophic cartilage	bone resorption	serum	RIA, ELISA

Abbreviations: PYD, pyridinoline; DPD, deoxypyridinoline; Gal-Hyl, galactosyl hydroxylysine; NTX, N-terminal crosslinked telopeptide of type I collagen; CTX, C-terminal crosslinked telopeptide of type I collagen; ICTP, carboxyterminal crosslinked telopeptide of type I collagen; TRACP 5b, tartrate-resistant acid phosphatase 5b; Cat K, cathepsin K; OC-f, osteocalcin fragments; BSP, bone sialoprotein; RIA, radioimmunoassay; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography, CLIA, chemoluminescence assay.

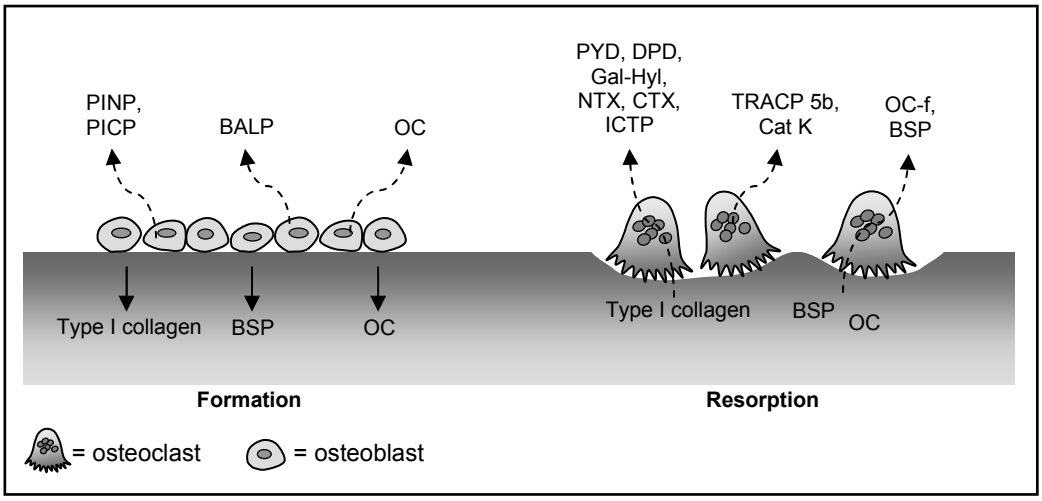

Figure 5. Biochemical origin of bone turnover markers.

2.3.1 Formation markers

Propeptides of type I collagen are extension peptides that flank the triple helical procollagen molecule. These extensions, referred to as the N- and C-terminal propeptides of type I collagen (PINP and PICP, respectively), guide the helical folding of the collagen molecule, and are cleaved by specific proteases, resulting in type I collagen formation and PINP and PICP release into the blood circulation. Both PINP and PICP are generated from newly synthesized collagen in a stoichiometric fashion, and therefore they can be considered quantitative measures of newly formed type I collagen. Type I collagen is also a component of several soft tissues, and therefore a potential contribution of circulating procollagens from soft tissue collagen synthesis exists. However, because the rate of collagen turnover in bone is much faster than in other tissues, the changes in procollagen concentrations are assumed to primarily reflect changes in bone collagen synthesis (Cremers et al. 2008). Immunoassays for both PINP and PICP have been developed (Melkko et al. 1990, Melkko et al. 1996, Taubman et al. 1974). In contrast to PINP, PICP demonstrates only a slight increase after menopause and correlates only weakly with spinal BMD. Therefore, PINP is considered a better measure of changes in the rate of collagen synthesis. Both PINP and PICP demonstrate a circadian rhythm with peak values occurring in the early morning.

Alkaline phosphatase (ALP) is a ubiquitous enzyme attached to glycosyl-phosphatidylinositol moieties located on the outer cell surface. Three tissue-specific genes encode the intestine, mature placenta, and germ-cell enzymes, whereas tissue non-specific ALPs are products of a single gene, and they differ after posttranslational modifications in their carbohydrate side chains (Weiss et al. 1988). The tissue non-specific forms are expressed in numerous tissues including bone, liver, kidney and early placenta. In bone, ALP is expressed on the cell surface of bone-forming osteoblasts, and it has been routinely used as a histochemical marker for osteoblasts. The precise function of the enzyme is still unknown, but it is likely to play an important role in osteoid formation and mineralization. The enzyme is probably cleaved off the membrane and released into the circulation where the enzyme activity

or mass can be detected by immunoassays. The two most common sources of elevated ALP levels are liver and bone. Immunoassays (Garnero and Delmas 1993, Gomez et al. 1995) using tissue-specific monoclonal antibodies to measure the bone specific isoform (BALP) still demonstrate 10-20% cross-reactivity with the liver isoform. Serum levels of BALP show no significant circadian variations.

Osteocalcin (OC) is a small hydroxyapatite-binding protein primarily synthesized by osteoblasts, but also by odontoblast, osteocytes and hypertrophic chondrocytes. It contains three vitamin K-dependent Gla residues responsible for the calcium binding properties of the protein (Hauschka et al. 1989). After its release from osteoblasts, most of the protein is incorporated into the extracellular bone matrix, while a smaller fraction is released into the circulation and can be detected by immunoassays. Circulating osteocalcin is constituted of different immunoreactive forms. Approximately one third of total osteocalcin in serum is intact, whereas the remainder is present as various fragments. The majority of the fragments exist as large N-terminal fragment 1-43, which represents one third of the circulating osteocalcin (Garnero et al. 1994). Most of the fragments are generated from the degradation of the intact molecule and, therefore, also reflect bone formation. In addition, differently carboxylated forms of OC exist, and the rate of γ-carboxylation is shown to affect fracture risk (Luukinen et al. 2000). Some osteocalcin fragments are also released during osteoclastic degradation of bone matrix (Ivaska et al. 2004). These will be discussed in the following section (see 2.3.2). Serum osteocalcin is considered as a sensitive and specific marker of osteoblast function, and numerous immunoassays using different antibodies have been developed. However, intact osteocalcin is rapidly degradated at room temperature, decreasing the reliability of the assay. The newer assays measuring both the intact molecule and its N-terminal fragment exhibit improved correlation with BMD.

2.3.2 Resorption markers

Pyridinoline (PYD) and deoxypyridinoline (DPD) are constituents in crosslinks of collagen, which form a covalent link between the collagen fibrils and mechanically stabilize the molecule. DPD is almost exclusively found in bone and dentin, whereas PYD is also expressed in cartilage, ligaments and vessels. During bone resorption the crosslinked collagens are proteolytically broken down and the crosslink components are released into the blood circulation and urine. Both markers demonstrate a strong circadian rhythm, with peak values in the early morning. High performance liquid chromatography (HPLC) has been widely used in the measurement of PYD and DPD. Although this method has been automated, it is still inconvenient, expensive and laborious. For routine use, simple and less expensive immunoassays have been developed to measure urinary PYD and DPD (Gomez et al. 1996, Robins et al. 1994). The measurement of hydroxypyridinium crosslink is not influenced by the degradation of newly synthesized collagens and, therefore, their level strictly reflects the degradation of mature crosslinked collagens (Robins 2001).

Galactosyl hydroxylysine (Gal-Hyl) is an integral part of bone collagen. It is also released into the circulation during bone degradation and has potential as a marker of collagen degradation. Another form of hydroxylysine glycosides, glucosyl-galactosyl-hydroxylysine (Clc-Gal-Hyl), is also released into the circulation during collagen

degradation, but it is considered less specific to bone collagen. Both Gal-Hyl and Clc-Gal-Hyl can be measured in urine by HPLC (Krane et al. 1977, Moro et al. 1984).

Cross-linked telopeptides of type I collagen include the cross-linked N-terminal (NTX) and C-terminal telopeptides of type I collagen (CTX and ICTP). They result from collagen fragmentation during bone resorption and are released into the blood circulation and urine. Generation of the fragments depends on different collagenolytic pathways. The NTX and CTX fragments are generated by Cat K, whereas the ICTP fragments are produced by matrix metalloproteinases (Atley et al. 2000, Garnero et al. 2003). CTX exists in an isomerized and a non-isomerized form referred to as β-CTX and α-CTX, respectively. The β-CTX contains an isoaspartyl (β-aspartyl) peptide bond in its L-enantiomeric form, which is considered to result mainly from ageing of extracellular proteins (Bonde et al. 1997). The isomerization process reaches a maximum approximately three years after the bone is mineralized, and therefore β-CTX reflects the degradation of relatively old bone (Fledelius et al. 1997). In contrast, the non-isomerized α-CTX presumably results from the degradation of relatively young bone. There are assays available for urinary α-CTX (Bonde et al. 1996) and β-CTX (Bonde et al. 1994) and for serum β-CTX (Bonde et al. 1997) and ICTP (Risteli et al. 1993). Presently, serum β-CTX is the most widely used crosslink assay and will be later referred as CTX. ICTP appears to be sensitive for pathological bone resorption as seen in multiple myeloma and metastatic bone disease. All the telopeptide markers are subject to major circadian variations.

Tartrate-resistant acid phosphatase 5b (TRACP 5b) is an enzyme secreted into the blood circulation by osteoclasts. Five acid phosphatase isoenzymes are detected in acidic polyacrylamide gel electrophoresis, of which only the most acidic, known as type 5 acid phosphatase, is resistant to tartrate inhibition, and is therefore also known as TRACP. Because of its abundant expression, TRACP has been routinely used as a histochemical marker of osteoclasts. However, the biological function of TRACP is still unclear. In osteoclasts, TRACP has been localized in intracellular lysosomal-like vacuoles and in transcytotic vesicles containing endocytosed bone matrix components (Halleen et al. 1999). In some studies it is also localized in the ruffled border area and in the resorption lacuna (Reinholt et al. 1990), suggesting that the enzyme may play a role in the process of bone resorption. Two closely related forms of TRACP circulate in human blood, TRACP 5a and TRACP 5b, of which TRACP 5b is derived from osteoclasts. TRACP 5b is secreted by both resorbing and non-resorbing osteoclasts into the circulation (Alatalo et al. 2000, Karsdal et al. 2003), and therefore it is considered as a marker of osteoclast number. TRACP 5b can be measured in the serum by recently developed immunoassays (Halleen et al. 2000, Janckila et al. 2004, Ohashi et al. 2007). Serum TRACP 5b levels exhibit very little circadian variation and are not influenced by food intake. TRACP 5b will be discussed in more detail in section 2.4.

Cathepsin K (Cat K) is a member of the cysteine protease family that, unlike other cathepsins, has the ability to cleave both helical and telopeptide regions of type I collagen (Garnero et al. 1998). It is secreted into the extracellular resorption lacuna, where it contributes to the cleavage of the collagen molecules and leads to degradation of the organic matrix. Thereafter, the bone degradation products and Cat K are endocytosed and transported through the cells (Vääräniemi et al. 2004) and released into the blood circulation, where they can be detected by immunoassays. Similar to TRACP 5b, Cat K is highly expressed in both resorbing and non-resorbing osteoclasts

(Henriksen et al. 2004, Karsdal et al. 2003), and considered as a potential marker of osteoclast number. Increased Cat K levels have been reported in patients with active rheumatoid arthritis, Paget's disease and in postmenopausal women with fragility fractures. Concentration of circulating Cat K is very low. Because currently available assays lack sensitivity, the accurate determination of serum Cat K remains challenging (Garnero 2008).

Urinary osteocalcin fragments (OC-f). Although circulating OC is widely used as an index of bone formation, some OC fragments are derived from the bone resorption process when OC is released from the bone matrix. These fragments are predominantly cleared from the blood circulation through glomerular filtration and accumulate in the urine. Hence, osteocalcin is more properly a marker of bone turnover rather than bone formation (Ivaska et al. 2004). Immunoassays for different fragments of urinary osteocalcin have been developed (Ivaska et al. 2005, Srivastava et al. 2002), and theoretically, urinary osteocalcin fragments may constitute a more specific bone resorption marker than type I collagen-related fragments, although their clinical value in osteoporosis remains to be more extensively evaluated (Garnero 2008).

Bone sialoprotein (BSP) is an acidic, phosphorylated and sialic acid-rich glycoprotein, which contains the RGD integrin binding site. BSP has been shown to be a major synthetic product of active osteoblasts, but it is also expressed by trophoblasts and it is strongly upregulated in a variety of human cancers (Fedarko et al. 2001). BSP accounts for 5-10% of the non-collagenous matrix proteins, and circulating BSP has been suggested to predominantly reflect processes related to bone resorption (Fohr et al. 2001, Seibel et al. 1996).

2.3.3 Assessment during growth and ageing

Throughout life, bone tissue is subject to a continuous process of turnover, whereby old bone is removed and replaced by new bone by the coupled process of bone resorption and formation. Increased bone turnover is associated with both bone increment and loss, and it is especially prominent during growth and senescence. These physiological conditions cause a significant increase in the serum concentrations of bone markers. In children, bone marker levels are elevated due to the rapid bone turnover during the skeletal growth and modeling, whereas in the elderly, the elevated marker levels result from an increase and negative balance in the remodeling process.

Research on bone turnover markers in children is increasing rapidly. A growing number of studies have assessed the reference intervals for several markers using various methods in healthy Caucasian and Irish school children (Table 2). Of these, two more recent studies have determined a large number of markers in the same pediatric population facilitating the comparison of the different markers (Rauchenzauner et al. 2007, van Coeverden et al. 2002). Rauchenzauner et al. established sex- and age-specific reference ranges for serum OC, BALP, CTX, ICTP and TRACP 5b in children of 0-18 years, enabling calculation of SD-scores. Van Coeverden et al. studied the association of serum BALP, OC, PINP, ICTP and urinary DPD with bone mass in boys and girls during puberty. In addition, age-dependent reference curves for serum BALP and PICP in healthy Chinese children (Tsai et al. 1999), for serum BALP in healthy Japanese children (Tobiume et al. 1997), for serum

Table 2. Studies assessing the pediatric reference intervals for bone turnover markers in healthy Caucasian or Irish school children.

Marker	Method	Age	n	Reference
S-PINP	RIA	Tanner 1, 2-3, 4-5	155	Lehtonen-Veromaa et al. 2000
	RIA	Tanner 1-5	306	van Coeverden et al. 2002
	RIA	0-19 years	334	Crofton et al. 2004
S-PICP	RIA	4-19 years	302	Crofton et al. 1997
S-BALP	ELISA	Tanner 1, 2-3, 4-5	155	Lehtonen-Veromaa et al. 2000
	ELISA	Tanner 1-5	306	van Coeverden et al. 2002
	EIA	Tanner 1-5	172	Fares et al. 2003
	IRMA	0-18 years	572	Rauchenzauner et al. 2007
S-OC	IRMA	1-16 years	1696	Cioffi et al. 1997
	RIA	Tanner 1, 2-3, 4-5	155	Lehtonen-Veromaa et al. 2000
	IRMA	Tanner 1-5	306	van Coeverden et al. 2002
	RIA	Tanner 1-5	172	Fares et al. 2003
	IRMA	0-18 years	572	Rauchenzauner et al. 2007
U-PYD/	HPLC	Tanner 1-5	176	Mora et al. 1998
U-DPD	HPLC	4-25 years	105	Rauch et al. 1994
	HPLC	6-19 years	240	Rauch et al. 2002
		Tanner 1-5	306	van Coeverden et al. 2002
U-NTX	ELISA	Tanner 1-5	176	Mora et al. 1998
S-CTX	ELISA	Tanner 1, 2-3, 4-5	155	Lehtonen-Veromaa et al. 2000
	ELISA	0-19 years	346	Crofton et al. 2002
	ELISA	Tanner 1-5	172	Fares et al. 2003
	ELISA	0-18 years	572	Rauchenzauner et al. 2007
S-ICTP	RIA	4-19 years	302	Crofton et al. 1997
	RIA	Tanner 1-5	306	van Coeverden et al. 2002
	RIA	0-28 years	572	Rauchenzauner et al. 2007
S-TRACP 5b	ELISA	0-18 years	572	Rauchenzauner et al. 2007
U-Gal-Hyl	HPLC	4-18 years	88	Rauch et al. 1995
	HPLC	6-19 years	240	Rauch et al. 2002

Abbreviations: S-, serum; U-, urine; PINP, N-terminal propeptide of type I collagen; PICP, C-terminal propeptide of type I collagen; BALP, bone specific alkaline phosphatase; OC, osteocalcin; PYD, pyridinoline; DPD, deoxypyridinoline; NTX, N-terminal crosslinked telopeptide of type I collagen; CTX, C-terminal crosslinked telopeptide of type I collagen; ICTP, carboxyterminal crosslinked telopeptide of type I collagen; TRACP 5b, tartrate-resistant acid phosphatase 5b; RIA, radioimmunoassay; ELISA, enzyme-linked immunosorbent assay; EIA, enzyme immunoassay; IRMA, immunoradiometric assay; HPLC, high-performance liquid chromatography.

OC in healthy Nigerian children (Oginni et al. 1996), and for serum TRACP 5b in healthy Chinese children (Chen et al. 2005) have been defined.

Despite the number of studies, the clinical application of the pediatric reference intervals is hindered because of variable results. The results may be affected by different analytic methods and biological variation, and some of the studies are limited in their relatively small number of subjects, incomplete coverage of age ranges and both genders, and less precise methodologies. However, the levels of bone turnover markers were in general found to be highest in the first two years of life, followed by a decrease during childhood, an increase again during puberty, and thereafter a decrease to the adult levels (reviewed by Yang and Grey 2006). The pubertal increases were 4-10 times the adult level and the magnitude of increase differed between the markers. During puberty, differences were observed in the marker levels of girls and boys, and the increased levels were observed later in boys. Assessment of a correlation between the markers and bone mass during puberty demonstrated significant correlations with one year change of BMD in girls (Lehtonen-Veromaa et al. 2000) and BMC in both sexes (van Coeverden et al. 2002). Interestingly, in the latter study, a significant correlation between bone mass and bone markers at baseline was observed in boys, but not in girls. It has been demonstrated that during puberty, sex, Tanner stage, whole-body mineral content, height velocity and whole-body mineral content accrual are significantly and independently associated with BALP and DPD, explaining 77-80% of the variability of these markers (Tuchman et al. 2008). Because of the complexity of bone metabolism in children, the marker results are more difficult to interpret than in adults, and bone marker concentrations can e.g. be similar in a child with high bone remodeling and low growth rate and in a normally growing child. In addition, the changes in bone marker levels may be influenced by many other factors, including vitamin D status and use of oral contraception. Hypovitaminosis D is frequent in children and adolescent, and serum 25-hydroxyvitamin D levels have been shown to affect the marker levels in children (Fares et al. 2003, Lehtonen-Veromaa et al. 2002, Viljakainen et al. 2006). Oral contraception may decrease bone turnover slightly, but it has in general little impact on the levels of the bone turnover markers (Cremers et al. 2008). In order to make most of the bone marker results and to interpret them correctly, the factors mentioned above should be taken into account.

Association of aging with the levels of bone turnover markers has been widely studied. In women, several large population-based studies have clearly shown age-related increases in serum BALP, OC, NTX and urinary PYD and DPD values (Delmas et al. 1993, Gallagher et al. 1998, Garnero et al. 1996b, Khosla et al. 1998, Lewis et al. 2000), and several other studies including additional markers have illustrated similar trends. Compared to premenopausal women, levels of both bone formation and resorption markers are substantially higher in postmenopausal women. The mean concentrations of urinary telopeptides are up to 100% higher and serum telopeptides 55-70% higher in postmenopausal women than in premenopausal women, whereas the formation markers are 20-50% higher (Garnero et al. 1996b). Although the marked increase in the marker levels begins to decline in elderly women, a higher bone turnover state is maintained for many years after menopause (Garnero et al. 1996b). In men, the effect of age on bone turnover markers is controversial. Both resorption and formation markers are reported to decrease with age and an additional small increase after the 5th decade has been observed in some studies. However, the magnitude of the

increase is of a much smaller degree compared to women. In contrast, other studies have shown an age-related increase in the marker levels without the initial decrease seen in studies mentioned earlier, and some studies have failed to show the effect of age on the marker levels (Henry and Eastell 2001).

2.3.4 Applications and limitations

Currently, biochemical markers are not used to diagnose osteoporosis. However, they have proven useful for monitoring treatment efficacy in postmenopausal osteoporosis and for improving fracture risk assessment. In addition, they are increasingly used during the preclinical and clinical development of drugs for treatment of metabolic bone diseases such as osteoporosis, Paget's disease and bone metastases. Presently, clinical application of bone markers in children are limited.

Monitoring of changes in bone with DXA is difficult because changes in bone mass are small and within the variation of DXA methodology. It may take 12-24 months before a significant change in BMD is reliably detected. Bone turnover markers change more rapidly and are sufficiently sensitive to effectively monitor the acute changes during therapy (Garnero et al. 1996a), and compared to BMD, the bone marker changes show a more favorable signal-to-noise ratio. Several studies have shown a significant inverse correlation between the short term decrease in the marker levels and a 2-3 year increase in BMD at various skeletal sites. Under adequate doses of estrogen, the mean decrease for various resorption markers varies from 20-60%. During bisphosphonate therapy, a significant decrease in resorption markers is seen already after one month of treatment and reaches a plateau from three months onwards. The decrease can be as much as 60-70% for the cross-link-related peptides CTX and NTX, and approximately 40-50% for DPD and TRACP 5b. The decrease in bone formation markers is delayed, reaching a plateau after 6-12 months of treatment (Delmas 2001). During treatment, a cut-off value of decrease can be defined for each marker as a value that will identify responders and non-responders with adequate sensitivity and specificity. Failure to respond to treatment may be due to non-compliance, which may be improved by monitoring the treatment efficacy with the marker measurements. For the development of new osteoporosis drugs, bone marker measurements can be used to establish an initial dose range (Miller et al. 2005). Bone turnover markers may also assist in the study of the physiology of skeletal growth and pathophysiology of diseases in childhood. Bisphosphonates are also used in children as therapy for primary bone diseases such as osteogenesis imperfecta, and increasingly for secondary osteoporosis caused by a variety of systemic diseases or their treatment. DPD has been particularly considered as an indicator of response to growth hormone therapy in children who have growth hormone deficiency (Schönau et al. 2001).

Increased biochemical markers of bone turnover can predict fragility fractures independently of age, BMD and prior fracture. This association has been assessed prospectively in several cohort studies and in case-control studies (reviewed by Szulc and Delmas 2008). Most of the data indicates that increased levels of bone resorption markers are predictive of vertebral, non-vertebral, hip and multiple fractures. In the same cohorts, bone formation markers were not associated with fracture risk, except BALP that predicted fracture risk (Garnero et al. 2000, Ross et al. 2000, Sornay-Rendu

et al. 2005). A recent long-term study investigating the incidence of fractures in elderly women for nine years concluded that elevated levels of serum TRACP 5b, CTX and urinary OC were consistently associated with increased vertebral fracture risk (Ivaska et al. 2007b). Thus, the combination of marker measurements and BMD can be used for improving the prediction of fracture. Early changes in bone marker levels are even shown to explain a larger proportion of the antifracture efficacy of bisphosphonates and raloxifene than changes in BMD (Bauer et al. 2004, Bjarnason et al. 2001, Eastell et al. 2003).

The limitations of the biochemical measurements of bone turnover markers include their analytical and pre-analytical variability, which affect the clinical interpretation of the marker levels. The analytical precision of both manual and automated assays is minimal, and therefore the major component of variability in bone marker levels is their preanalytical variability. Uncontrollable sources of preanalytical variability include age, gender, menopausal status, drugs and recent fracture, whereas the controllable sources include circadian, menstrual, exercise and feeding effects (Cremers et al. 2008). Circadian variability has probably more impact than any other factor. Most of the resorption and formation markers exhibit significant circadian rhythms increasing at night, reaching a peak between 01.30 - 08.00 and falling during the day, being lowest in the afternoon (Blumsohn et al. 1994, Eastell et al. 1992). The amplitude of the rhythm is greater for the resorption markers than for the formation markers. For serum CTX and urinary DPD it may be as much as 80% of the 24 h mean, whereas for PICP and OC it is 5-20%. Because of the large day-to-day variation, urinary resorption markers are generally reported after normalizing to creatine excretion. The variability is generally smaller for markers measured from serum samples compared to urine samples. Fracture healing (Ivaska et al. 2007a) and bed rest (Inoue et al. 2000) also have a significant increasing effect on the marker levels. Intake of food has been shown to decrease the levels of some markers, and therefore sample collection in the fasting state is preferable. Several markers are sensitive for thermodegradation, hemolysis and repeated freeze-thaw cycles. Therefore, storage of serum samples in several aliquots in a temperature of at least -70°C is recommended.

Although bone markers are usually classified as formation and resorption markers, some markers may reflect, at least to a certain degree, both formation and resorption. Both physiological and pathological conditions affect the serum concentration of bone markers, and most of the markers are also present in tissues other than bone and may therefore be influenced by non-skeletal processes as well. In addition, changes in marker levels are usually not disease-specific, but reflect alterations in skeletal metabolism independent of the underlying cause. Presently, the markers are measured individually by manual or automated immunoassays, or by chromatographic assays such as HPLC or LC/MS. Radioimmunoassays have limited routine clinical application because of safety issues. Although the contribution of analytical imprecision to overall variability in bone marker levels is relatively small, substantial differences can exist among different methods for the same marker. This may be relevant for relating results obtained with one method to data obtained with another method (Cremers et al. 2008).

2.4 Tartrate-resistant acid phosphatase (TRACP)

2.4.1 Introduction

Enzymes are proteins that function to catalyze chemical reactions in biological systems. They convert specific substrates to products without being consumed themselves, and therefore are able to catalyze subsequent reactions. Enzymes are classified according to their reaction and substrate specificity into oxido-reductases (EC 1), transferases (EC 2), hydrolases (EC 3), lyases (EC 4), isomerases (EC 5) and ligases (EC 6). Hydrolases can be further classified into esterases (EC 3.1), and even further into phosphatases (EC 3.1.3). The acid phosphatases (EC 3.1.3.2) found in human include lysosomal, prostatic, erythrocytic, macrophage and osteoclastic acid phosphatases (Bull et al. 2002). Of these, the macrophage and osteoclastic acid phosphatases were demonstrated to be resistant to inhibition by tartrate, and subsequently referred to as tartrate-resistant acid phosphatase (TRACP).

TRACP is also referred to as type 5 acid phosphatase, purple acid phosphatase (PAP) and uteroferrin, and has previously been abbreviated as TRAP. The enzyme was discovered and partially purified from bovine spleen (Sundararajan and Sarma 1954), and characterized by its purple color and acidic pH optimum. The name type 5 acid phosphatase refers to early studies that revealed five main bands of acid phosphatases in acidic polyacrylamide gel electrophoresis (Li et al. 1970). The bands were numbered according to their electrophoretic mobility towards the cathode, and only the most cathodal band 5 was tartrate-resistant (Li et al. 1973). The lysosomal (bands 1 and 3) and prostatic (bands 2 and 4) acid phosphatases are sensitive to inhibition by tartrate (Moss 1986). Other tartrate-resistant acid phosphatases exist in platelets and erythrocytes, but they are not demonstrable in the acidic polyacrylamide gels. Together with similar enzymes isolated from mammals, plants and micro-organisms, TRACP belongs to the group of purple acid phosphatases. The name purple acid phosphatase refers to the unique purple color of the enzyme, whereas uteroferrin refers to studies where TRACP was purified from the uterine fluids of pigs (Schlosnagle et al. 1974).

TRACP has been purified from various mammalian sources, including pig uterine fluid, spleens of patients affected with hairy cell leukemia and Gaucher's disease, bovine and rat spleen, human, rat and bovine bone, and human lungs and placenta (reviewed by Oddie et al. 2000).

2.4.2 Gene

Mammalian TRACP genes have been cloned from human (Cassady et al. 1993, Fleckenstein et al. 1996, Ketcham et al. 1989, Lord et al. 1990), mouse (Cassady et al. 1993, Reddy et al. 1993), rat (Ek-Rylander et al. 1991b) and pig (Vallet and Fahrenkrug 2000). In human, a single gene encoding TRACP has been localized to chromosome 19p13.2-13.3 (Leach et al. 1994, Lord et al. 1990), whereas in murine the gene is located in a syntenic region of chromosome 9 (Grimes et al. 1993). The exon-intron structure of the gene is highly conserved, consisting of five exons with the initiation signal at the beginning of exon 2. Studies of the murine TRACP mRNA suggest that the expression is regulated by the use of four alternative tissue- and cell-restricted promoters (Walsh et al. 2003). The promoter area contains numerous

putative transcription factor binding sites, including PU.1, Sp1, AP1, GT-1 and H-APF-1 (Reddy et al. 1993). Of these, PU.1 is shown to cooperate with the microphthalmia transcription factor (MiTF). MiTF binds directly to the proximal TRACP promoter and co-operates with PU.1, which binds to an adjacent region of the promoter, increasing TRACP promoter activity (Luchin et al. 2001). In addition to these, a putative iron responsive element has been found in uteroferrin (Simmen et al. 1989), and TRACP expression has been shown to be decreased by the iron-delivering agents hemin and protoporphyrin IX (Reddy et al. 1996), but to be increased by transferrin (Alcantara et al. 1994). The TRACP cDNA cloned from human contains 1359-1412 nucleotide sequence with an open reading frame of 969-975 bp leading to 323-325 amino acids (Ketcham et al. 1989, Lord et al. 1990). mRNA of approximately 1.5 kb is translated into a single polypeptide (Hayman et al. 1991).

2.4.3 Protein structure

Mature human TRACP consists of 304 amino acids, with an unglycosylated relative molecular weight of 34,193 (Ketcham et al. 1989, Sträter et al. 2005). The sequence contains two potential N-glycosylation sites (Asn95 and Asn126), seven residues coordinating two Fe atoms (Asp12, Asp50, Tyr53, Asn89, His184, His219 and His221), and a Cys(X)$_5$Arg catalytic motif (Cys198-Arg204) essential for phosphoester hydrolysis (Sträter et al. 2005, Zhang et al. 1994) (Figure 6).

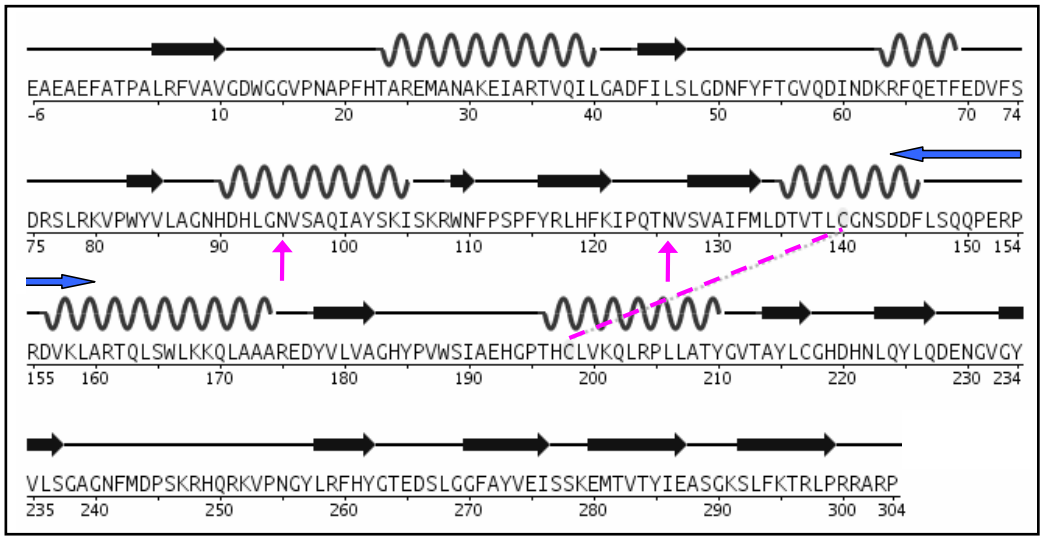

Figure 6. Amino acid sequence and secondary structure assignment of human recombinant TRACP. Disulfide bond between Cys140 and Cys198 and two putative N-linked glycosylation sites (Asn95 and Asn126) are highlighted in purple line and arrows, respectively. The sequence of the repression loop is marked with blue arrows. β-sheets and α-helices of the secondary structure are represented above the amino acid sequence [Obtained from Protein Data Bank (PDB), http://www.rcsb.org/pdb/, PDB ID 1war].

The crystal structures of several kidney bean PAPs [Protein Data Bank (PDB) IDs 1kbp, 3kbp and 4kbp, released 1996, and 2qfr and 2qfp, released 2008], uteroferrin (pig TRACP; 1ute, released 1999), rat TRACP from bone (1qhw, released 1999), recombinant rat TRACP (1qfc, released 2000), sweet potato PAP (1xzw, released 2004) and recombinant human TRACP (1war and 2bq8, released 2005) have been determined. The molecular weight of mammalian TRACPs is approximately 35 kD, whereas plant PAPs are typically 110 kD homodimers. Despite the low overall sequence homology, the core structure of both plant PAPs and mammalian TRACPs is very similar, containing seven metal coordinating amino acids in the catalytic site. The catalytic site contains a trivalent-divalent dinuclear metal center, where the trivalent ion is Fe^{3+}. The divalent ion is Fe^{2+} in mammalian TRACPs and either Zn^{2+} or Mn^{2+} in plant PAPs. The characteristic purple color of the enzyme arises from a tyrosine to Fe^{3+} charge transfer transition. The divalent ferrous ion (Fe^{2+}) in mammalian TRACPs is redox active, and can be oxidized to result in diferric Fe^{3+}-Fe^{3+} form. Asp50 (according to human sequence) bridges the two iron ions together with a water based (hydr-)oxo ligand.

Mammalian TRACPs consist of two seven-stranded β-sheets surrounded on both sides by α-helices. The two β-sheets pack onto each other, forming a β-sandwich, and the binuclear iron center is located at the bottom of the active site pocket at one edge of the β-sandwich. A disulfide bond located between the residues Cys140 and Cys198 (according to the human sequence) is observed in the structures of uteroferrin (1ute), rat bone TRACP (1qfc) and human recombinant TRACP (1war and 2bq8). At the vicinity of the active site resides an exposed, highly antigenic repression loop formed by residues 145-160 (according to human sequence), which is susceptible for proteolytic processing (Figures 6 and 7). TRACPs purified from human and rat bone (Ek-Rylander et al. 1991a, Halleen et al. 1996), giant cell tumors (Hayman et al. 1989) and spleen (Ketcham et al. 1985, Orlando et al. 1993, Robinson and Glew 1980b) are obtained in a proteolytically cleaved form consisting of N-terminal 20-23 kD and C-terminal 15-17 kD fragments linked by a disulfide bond, whereas uteroferrin (Ketcham et al. 1985, Ling and Roberts 1993) and recombinant TRACPs expressed in *Baculovirus* are purified as a single polypeptide (Ek-Rylander et al. 1997, Hayman and Cox 1994, Marshall et al. 1997).

The repression loop has an important role in regulating enzyme activity. The cleaved two-subunit structure displays increased enzymatic activity. Furthermore, cleavage at the end of the loop, near the sequence ^{160}ARTQ, seems to produce proteins with higher activity than cleavage upstream of this sequence (Funhoff et al. 2001a). Mutational analysis further specified that interaction of loop residue Asp144 (according to human sequence) with the active site residues is a major factor regulating the catalytic activity (Funhoff et al. 2001b). In addition, the crystal structures of human recombinant TRACP expressed in *Escherichia coli* and *Pichia pastoris* show that the repression loop is flexible and can adopt a conformation that blocks the active site and abolishes the catalytic activity (Sträter et al. 2005). Thus, the conformation of the loop and its proteolytical cleavage may constitute a potential physiological mechanism for regulating the enzyme activity.

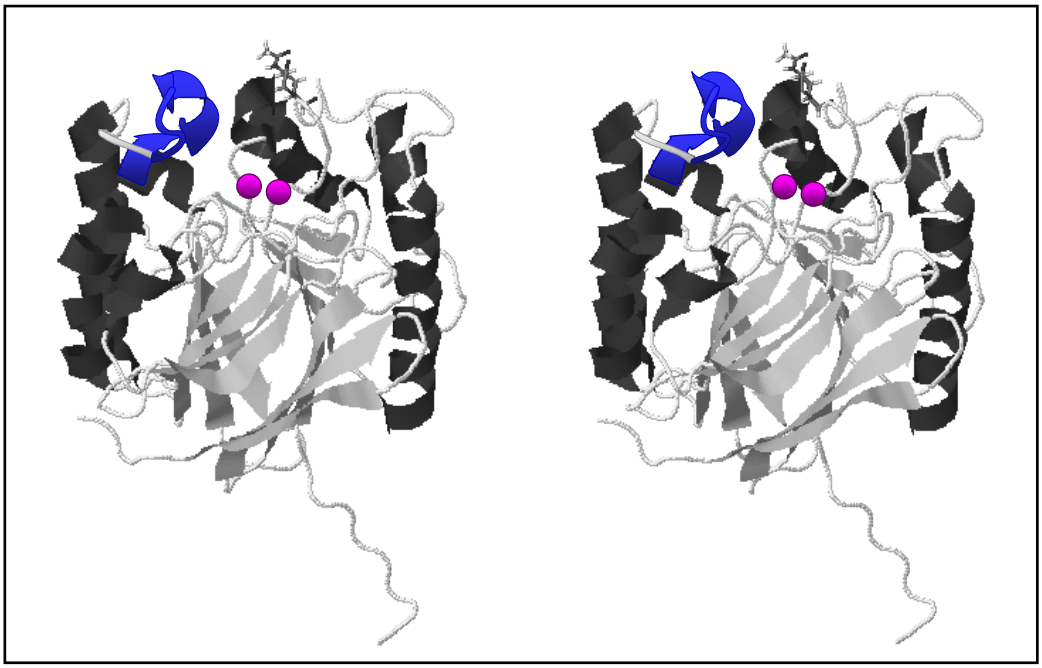

Figure 7. Stereo view of human recombinant TRACP. Fe atoms are highlighted in purple, and the repression loop is shown in blue. A single carbohydrate residue (NAG) is bound to Asn95. Six amino acid cloning artifact at the N terminus is incorporated into the protein by the *Pichia* expression system (Obtained from PDB, PDB ID 1war, modified using Jmol: an open-source Java viewer for chemical structures in 3D, http://www.jmol.org/).

Despite the presence of two possible sites for N-linked glycosylation (Asn95 and Asn126, according to human sequence), most studies and crystal structures demonstrate glycosylation only at Asn95. However, rat recombinant TRACP expressed in *Spodoptera frugiperda 9 (Sf9)* cells is shown to be glycosylated at both potential sites (Wang et al. 2005), suggesting that the glycosylation at Asn126 may also be present but not detected in the crystallized proteins. The sugar chains consist of a high-mannose-type oligosaccharide ($Man_{5-6}GlcNAc_2$) chain (Saunders et al. 1985) and multi-antennary complex type chains (Kawaguchi et al. 2008), and some of them contain sialic acid residue (Lam et al. 1978a). In addition, a paucimannosidic ($Man_{3-4}GlcNAc_2$) chain may be present (Wang et al. 2005). Interestingly, differential glycosylation has also been reported to regulate the enzymatic properties (Wang et al. 2005), but it could also lead to targeting of the enzyme to different subcellular locations.

2.4.4 Expression

Expression of TRACP is widely distributed in normal tissues. Both the mRNA and protein are expressed abundantly in bone, spleen, liver, the linings of the gastrointestinal tract, lung, thymus and skin. The phosphatase activity of TRACP is highest in bone, spleen, liver, thymus and colon, and lower in lung, stomach, skin, brain and kidney (Hayman et al. 2000b). The relative distribution of mRNA level is

shown to be comparable to that of enzyme activity, except in bone and lung. In bone, the relative expression of enzyme activity is considerably higher than the mRNA level, whereas the opposite pattern is observed in the lung (Lång and Andersson 2005), indicating that the enzyme is much more active in bone than in lung. Moreover, in bone and liver, the expression was shown to decline with advancing age, whereas in the lymphoid organs (spleen and thymus), the level of expression was constant or even increased (Lång and Andersson 2005).

In bone, the expression of TRACP is mainly restricted to osteoclasts (Minkin 1982). However, the enzyme has been also detected in osteoblasts and osteocytes (Bianco et al. 1988, Yamamoto and Nagai 1998). In lung, TRACP has been shown to be expressed by alveolar macrophages, and in the liver, the expression is detected in a specialized macrophage progeny of Kupffer cells (Efstratiadis and Moss 1985, Yaziji et al. 1995). Apart from bone, lung and liver, the detection of TRACP in diverse tissues is primarily due to the wide distribution of dendritic cells (Hayman et al. 2000a). The placenta seems to be unique in that TRACP is expressed primarily by decidual cells and syncytio-trophoblasts that are not of macrophage origin (Janckila et al. 1996).

Elevated TRACP expression occurs in pathological conditions such as hairy cell leukemia, Gaucher's disease and osteoclastoma (Hayman et al. 1989, Robinson and Glew 1980a), and TRACP has been used as a histochemical marker of these diseases. Increased TRACP activity has also been demonstrated in the serum of patients with Paget's disease and hyperparathyroidism (Halleen et al. 2001, Stepan et al. 1983), and in inflammatory conditions such as rheumatoid arthritis (Chao et al. 2005a, Janckila et al. 2002). A marked increase in serum TRACP concentrations is observed in patients with breast and ovarian cancer, regardless of the presence or absence of bone disease. Furthermore, TRACP expression has been found in tissues of breast and ovarian cancer and in malignant melanoma (Adams et al. 2007, Honig et al. 2006).

2.4.5 Subcellular localization

In osteoclasts, TRACP is localized at the ultrastructural level in cytoplasmic vesicles or vacuoles of various sizes, and in small dense granules in the mitochondria-rich part of the cytoplasm (Clark et al. 1989). Moreover, TRACP co-localizes with intracellular vesicles containing Cat K and endocytosed bone degradation products released from bone matrix during resorption (Halleen et al. 1999, Vääräniemi et al. 2004). In these studies, TRACP was not observed at the ruffled border area or in the resorption lacuna. However, another study has demonstrated TRACP at the ultrastructural level in the ruffled border area and in the resorption lacuna (Reinholt et al. 1990).

In activated macrophages, TRACP is partially co-localized with late endosomal/ lysosomal markers Rab7, Lamp1 and MHCII, and with internalized *Staphylococcus aureus* (Räisänen et al. 2001). In some studies, TRACP has also been detected in osteoblasts. At the ultrastructural level, osteoblastic TRACP is detected in lysosomes and in the Golgi complex, including lamellae, vesicles and vacuoles described as primary lysosomes and/or secretory granules (Yamamoto and Nagai 1998). However, some studies have not detected TRACP in these cells (Clark et al. 1989, Reinholt et al. 1990).

2.4.6 Isoforms 5a and 5b

Two isoforms of TRACP exist in human serum. These isoforms are referred to as TRACP 5a and TRACP 5b according to their electrophoretic mobility (Lam et al. 1978a). The difference in mobility was shown to be due to an additional sialic acid residue in the carbohydrate side chain of the 5a form (Lam et al. 1981). Later it was discovered that the 5a form consists of a single polypeptide, whereas the 5b form consists of two subunits (Janckila et al. 2003). In addition, TRACP 5a was shown to mainly contain a high-mannose type sugar chain, whereas TRACP 5b has a multi-antennary complex type sugar chain (Kawaguchi et al. 2008). The isoforms are products of the same gene, indicating that the 5a and 5b forms result from differential posttranslational processing.

As a result of the post-translational modifications, TRACP 5a and 5b possess different enzyme activity and pH optimum, and they have distinct preferences for synthetic substrates. The 5b form exhibits approximately 5.5-fold higher specific activity than the 5a form (Janckila et al. 2001a). The pH optimum is 5.0-5.2 for TRACP 5a and 5.8 for TRACP 5b (Lam et al. 1978a). From the synthetic substrates, naphtol-ASBI phosphate (N-ASBI-P) and 2-chloro-4-nitrophenyl phosphate (CNPP) are preferred by the 5b form (Janckila et al. 2001b, Ylipahkala et al. 2008). The major factor contributing to the differences between TRACP 5a and 5b seems to be proteolytic excision of the repression loop (Janckila et al. 2005), which results in the two subunit structure with increased enzyme activity and altered pH optimum. The differential properties of the 5a and 5b forms are summarized in Table 3.

Table 3. Differences between TRACP 5a and TRACP 5b.

Property	TRACP 5a	TRACP 5b
Structure	Monomeric, 35 kD	Two subunits, 16 + 23 kD
Glycosylation	High-mannose type, contains sialic acid	Multi-antennary complex type
Specific activity	Low (0.58 U/μg)	High (3.30 U/μg)
pH optimum	5.0 – 5.2	5.8
Predominance in tissue expression	Lung, placenta	Bone, spleen, colon, kidney, liver, thymus
Secreted by	Macrophages, dendritic cells	Osteoclasts

In rat tissues, the 5b form predominates in bone, spleen, colon, liver and thymus, whereas the 5a form predominates in lung (Lång and Andersson 2005). At the cellular level, human osteoclasts, macrophages and dendritic cells are shown to contain both TRACP 5a and 5b, but exhibit differences in the secretion of the isoforms. Osteoclasts appear to mainly secrete the 5b form, and the 5a form is most likely secreted by macrophages and dendritic cells (Janckila et al. 2005). The 5a form may represent a latent proenzyme, which is activated by proteolytical processing, or more likely the 5a and 5b forms represent two functionally different end products of the same gene with differential tissue- and cell specific expression.

In the circulation, approximately 90% of TRACP exists in 5a form, whereas the remaining 10% represents the 5b form (Janckila et al. 2001a). Due to the higher specific activity of the 5b form, both isoforms represent approximately 50% of the circulating TRACP activity. Immunoassays for measuring TRACP 5b activity and TRACP 5a activity and amount have been developed (Chao et al. 2005a, Halleen et al. 2000). Elevated TRACP 5b activity in serum is associated with bone turnover, whereas elevated serum TRACP 5a level is associated with conditions of chronic inflammations. The assessment of serum TRACP 5a and 5b will be discussed in more detail in sections 2.4.9.2 and 2.4.9.3.

2.4.7 Enzyme activity

TRACP possesses two distinct enzyme activities; an acid phosphatase (AcP) and reactive oxygen species (ROS) generating activity. Both activities utilize the same redox-active iron at the active site, but they are independent of each other and have different pH optima (Kaija et al. 2002), suggesting that the two activities may function in different cellular compartments, and the pH environment may determine which activity is used.

2.4.7.1 Acid phosphatase

In an acidic environment, TRACP can catalyze the hydrolysis of a wide range of natural and synthetic phosphate esters. The enzyme is able to hydrolyze aromatic phosphoesters such as 4-nitrophenyl phosphate, α-naphtyl phosphate and phospho-tyrosine, and phosphoanhydrides such as pyrophosphate and nucleoside tri- and diphosphates (Anderson and Toverud 1986, Janckila et al. 1992, Schlosnagle et al. 1974). Aliphatic phosphoesters such as β-glycerophosphate, phosphoserine and phosphothreonine are less efficiently hydrolyzed (Lau et al. 1985). The activity can be enhanced by mild reducing agents such as β-mercaptoethanol, dithiotreitol and ascorbate (Davis et al. 1981, Schlosnagle et al. 1974). Of these, β-mercaptoethanol and dithiotreitol reduce the disulphide bond, whereas ascorbate reduces the redox-active ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) with concomitant change in color from purple to pink. The activity can be competitively inhibited by inorganic phosphate and its analogs such as vanadate, arsenate and molybdate, and non-competitively inhibited by fluoride, copper and zink (Anderson and Toverud 1986, Janckila et al. 1992, Schlosnagle et al. 1974). Oxidizing agents such as hydrogen peroxide deactivate TRACP by converting the Fe^{2+} to Fe^{3+}, whereas strong reducing agents such as dithionite irreversibly deactivate the enzyme by removing the iron center, resulting in a colorless form (Schlosnagle et al. 1974). A major factor regulating AcP activity is the repression loop of the enzyme. Excision of the loop peptide results in a two-subunit structure with enhanced activity and altered catalytic mechanism (Funhoff et al. 2005, Ljusberg et al. 1999). Spectroscopical studies suggest that the phosphate group of the substrate binds to the Fe^{2+} of the proteolytically cleaved form of the enzyme, but not to that of the single polypeptide form. This is followed by a nucleophilic attack on the phosphorus and release of the product. Possible candidates for the attacking nucleophile include (1) a terminal hydroxide bound to the Fe^{3+}, (2) a terminal hydroxide bound to the Fe^{2+}, (3) a hydroxide bridging the metal ions, and (4) a water in the second coordination sphere of the Fe^{3+} (Funhoff et al. 2005).

2.4.7.2 Generator of reactive oxygen species

A neutral environment is optimal for the ROS generating activity of TRACP. Free radical formation is catalyzed by redox-active iron and proceeds through the Fenton reaction (Halleen et al. 1999, Hayman and Cox 1994, Sibille et al. 1987). In the mixed valent form (Fe^{3+}-Fe^{2+}) of the enzyme, Fe^{2+} is able to react with hydrogen peroxide and produce Fe^{3+} and hydroxyl radical ($OH^{\bullet}$) (Equation 1). The Fe^{3+} produced is able to further react with hydrogen peroxide, regenerating the Fe^{2+} and forming a superoxide anion ($O_2^{\bullet}$) (Equation 2). A sequence of reactions generating both hydroxyl radicals and superoxide anions can continue as long as hydrogen peroxide is available.

(1) $\text{TRACP-Fe}^{3+}\text{-Fe}^{2+} + H_2O_2 \rightarrow \text{TRACP-Fe}^{3+}\text{-Fe}^{3+} + OH^{\bullet} + OH^-$

(2) $\text{TRACP-Fe}^{3+}\text{-Fe}^{3+} + H_2O_2 \rightarrow \text{TRACP-Fe}^{3+}\text{-Fe}^{2+} + O_2^{\bullet -} + 2H^+$

2.4.8 Proposed functions

Increasing number of studies have assessed the function and importance of TRACP both *in vitro* and *in vivo*. Mouse models for TRACP deficiency (Hayman et al. 1996) and TRACP overexpression (Angel et al. 2000) have been generated. Furthermore, a mouse strain lacking both TRACP and lysosomal acid phosphatase (LAP) has been generated (Suter et al. 2001) by breeding the TRACP knock-out mice with LAP knock-out mice (Saftig et al. 1997).

The TRACP knock-out mice were generated by targeted gene disruption. Despite the total lack of TRACP, both the TRACP and TRACP/LAP double knock-out mice are viable and fertile, but weigh approximately 10-15% less than control or LAP knock-out mice. TRACP overexpressing mice were generated by introducing additional copies of the gene with an SV40 enhancer element into the mouse genome. A copy number-dependent increase in TRACP mRNA level and enzyme activity was observed with high levels of the mRNA expression in the liver, kidney and spleen, and smaller amounts in the heart, brain and lung. The inclusion of the transgene did not result in TRACP activity at abnormal sites, but it resulted in increased enzyme activity. TRACP activity in the heart, spleen, lung, brain and kidney was restricted to macrophage-like cells. In the pancreas, TRACP activity was expressed specifically in the secretory acinar cells.

2.4.8.1 Role in bone resorption

The most striking abnormality observed in both the TRACP deficient and overexpressing mice is their skeletal phenotype, suggesting that TRACP has a role in bone metabolism. The knock-out mice have progressive foreshortening and deformity of the long bones and axial skeleton, suggesting a role for TRACP in endochondral ossification. Older animals have higher mineral density in the long bones, and osteoclasts isolated from the mice resorb approximately 25% less efficiently than wild-type littermates. An osteopetrotic phenotype is apparent at four weeks of age (Hayman et al. 1996). The formation of osteoclasts is normal, suggesting that TRACP is not essential during osteoclastogenesis. However, the osteoclasts formed exhibit an increased relative area of ruffled border and accumulation of cytoplasmic vesicles

containing filamentous material (Hollberg et al. 2002). The mice were shown to have abnormal type I collagen and mineral metabolism in bone, associated with altered resistance to fracture due to mechanical stress (Roberts et al. 2007b). TRACP/LAP double knock-out mice display marked alterations in soft and mineralized tissues. They are characterized by a progressive hepatosplenomegaly, gait disturbances and exaggerated foreshortening of the long bones (Suter et al. 2001). Overexpression of TRACP caused significantly decreased levels of trabecular bone, and conversely, a more than two-fold increase in the bone formation rate, indicating a higher rate of bone turnover. No change in the number of osteoclasts was observed, but the cells exhibited more intense TRACP activity staining (Angel et al. 2000). The results suggest that individual osteoclasts have an increased resorption rate, which is compensated by increased bone formation, resulting in relatively little net bone loss and mild osteoporosis.

Although functional studies have demonstrated that TRACP is able to dephosphorylate phosphotyrosine, it has not been co-localized with phosphotyrosine containing substrates. Instead, co-localization is observed with phosphoserine containing bone matrix proteins osteopontin (OPN), and bone sialoprotein (BSP), which are also dephosphorylated by TRACP *in vitro* (Ek-Rylander et al. 1994). Both OPN and BSP contain the RGD motif, and are thus possibly involved in osteoclast attachment to the bone surface. Dephosphorylation of OPN has been shown to impair its ability to promote adhesion as well as migration of osteoclasts *in vitro* (Andersson et al. 2003). The results suggest that TRACP could function as OPN phosphatase, regulating both cell adhesion and migration. However, in some studies TRACP has not been observed in the resorption lacuna (Clark et al. 1989, Halleen et al. 1999), thus creating a debate of whether the dephosphorylation of osteopontin by TRACP in the lacuna can or can not occur. As an intracellular protein, TRACP could function as a protein-tyrosine phosphatase regulating protein-tyrosine kinases, and thereby affect various cellular events such as signal transduction, proliferation and differentiation (Janckila et al. 1992, Nuthmann et al. 1993).

In addition to the acid phosphatase function, a role for the ROS generating activity of TRACP in osteoclasts has been suggested. Intracellular TRACP is co-localized with bone degradation products and Cat K in transcytotic vesicles (Vääräniemi et al. 2004). Consequently, TRACP has been suggested to function as a generator of ROS in these vesicles, thus playing a role in the further degradation of the internalized bone matrix components (Halleen et al. 1999). In general, hydroxyl radicals in combination with superoxide anions are highly destructive, causing protein fragmentation (Davies and Delsignore 1987), and the free radicals generated by TRACP have been shown to be capable of destroying type I collagen *in vitro*, without disruption of the enzyme itself (Halleen et al. 1999). The hypothesis is supported by the finding that part of the transcytotic vesicles exhibit neutral pH (Vääräniemi et al. 2004), which is favorable for ROS generation. Furthermore, both hydrogen peroxide and superoxide, compounds required for the Fenton reaction, can be produced by the resorbing osteoclast (Steinbeck et al. 1994).

Accumulation of cytoplasmic vesicles containing filamentous material observed in osteoclasts of TRACP knock-out mice (Hollberg et al. 2002) could result from incomplete digestion of internalized bone matrix components, consistent with the idea of TRACP involvement in their degradation by catalyzing the ROS production.

Alternatively, accumulation of the vesicles could result from a slower rate of intracellular vesicular trafficking, possibly regulated by TRACP mediated dephosphorylation of phosphoproteins (Hollberg et al. 2002). However, the accumulated vesicles are not likely to be derived from a secretory pathway, since Cat K was detected at normal levels in the ruffled border area and bone matrix.

Osteoclasts express both TRACP 5a and 5b, but they predominantly secrete the 5b form (Janckila et al. 2005). Two pathways for secretion of TRACP have been proposed. One hypothesis is that TRACP-containing acidic vesicles fuse with the transcytotic vesicles in which TRACP is cleaved and subsequently activated by Cat K. During the transcytosis, this TRACP 5b form generates ROS that further degrade the internalized matrix components. The transcytotic vesicles fuse with the FSD and both TRACP 5b and the remaining bone degradation products are released into the circulation (Vääräniemi et al. 2004). The other hypothesis is that TRACP is transported in the secretory pathway and secreted in the ruffled border area where Cat K processes it to the active 5b form. The active enzyme accumulates in the bone matrix and is partly internalized along with the bone matrix components followed by transcytosis and secretion through FSD concomitant with the former hypothesis (Ljusberg et al. 2005). The circulating TRACP 5b released from the osteoclasts can be used as a marker of osteoclast number, which will be discussed in more detail in section 2.4.9.3.

Interestingly, attempts to knock down the TRACP expression in osteoclasts and monocytes by antisense DNA and RNA interference methods have resulted in increased TRACP expression, suggesting that the enzyme expression may have a role in pathogen recognition and in innate immunity (Muhonen et al. 2007).

2.4.8.2 Role in immune system

TRACP has been shown to affect macrophage and dendritic cell function, and it may play a role in the development of autoimmune diseases. Macrophages and dendritic cells express both TRACP 5a and 5b. The 5a form is predominantly secreted, whereas both 5a and 5b have been observed intracellularly (Janckila et al. 2005). Macrophages from the TRACP knock-out mice display an increased production of nitric oxide, superoxide and proinflammatory cytokines in response to natural activators, suggesting a role for TRACP in innate immunity (Bune et al. 2001). The mice also display reduced clearance of the microbial pathogen *Staphylococcus aureus* after sublethal intraperitoneal inoculation. However, the peritoneal macrophages and neutrophils are able to normally phagocytose and kill *Staphylococcus aureus in vitro*, suggesting that TRACP would influence recruitment of macrophages to the site of microbial invasion. The macrophages also demonstrate an increase in tartrate-sensitive LAP, suggesting that TRACP and LAP may have overlapping functions. In TRACP overexpressing mice, the macrophages exhibit increased production of both ROS and superoxide, and the cells also show a tendency for increased bacterial killing (Räisänen et al. 2005). Studies in murine macrophage-like cell line RAW-264 overexpressing TRACP demonstrate co-localization of TRACP, internalized *Staphylococcus aureus* and MHCII molecules, and increased intracellular ROS production (Räisänen et al. 2001). The results indicate that TRACP is localized in the antigen presentation route transporting endocytosed foreign material into the cell membrane where they are presented to other cells of the

immune system, and the ROS generated by TRACP have been suggested to play a role in antigen processing (Halleen et al. 1999).

Dendritic cells from the TRACP knock-out mice display impaired maturation and reduced Th1 responses (Esfandiari et al. 2006). The ability of LPS to up-regulate the expression of MHCII and CD80 in dendritic cells was reduced compared to wildtype mice, although production of IL-10 was increased. The mice displayed a delayed response to sensitization with picryl chloride and reduced T cell proliferation after immunization with ovalbumin. In addition, reduced ovalbumin-specific IgG2a production was observed. The findings demonstrate that TRACP has no intrinsic role in T and B cell development and function, but it has a role in the process of dendritic cell maturation. The results suggest that TRACP is important for polarizing responses in naïve T cells to antigen-presenting dendritic cells.

Overactivation of the Th1 pathway by macrophages or dendritic cells can generate organ-specific autoimmune diseases such as arthritis, multiple sclerosis and type I diabetes. Both TRACP and its possible substrate osteopontin have been implicated in the development of rheumatoid arthritis (RA). An approximately two fold increase of total TRACP activity has been observed in the synovial fluid of arthritic diseases seropositive for rheumatoid factor compared to those with seronegative arthritic disease (Luukkainen et al. 1990), and an increased amount of total TRACP and TRACP 5a have been observed in the serum of patients with RA (Chao et al. 2005a, Janckila et al. 2001a). Consequently, the use of circulating TRACP 5a as a biochemical marker of chronic inflammation is being studied, which will be discussed in more detail in section 2.4.9.2. It has also been demonstrated that TRACP activity is inhibited by therapeutic gold (Hayman and Cox 2004), a disease modifying agent that affects antigen processing by T cells and is used for the treatment of RA. Furthermore, studies using osteopontin deficient mice have demonstrated that the absence of osteopontin protects joints against destruction in anti-type II collagen antibody-induced arthritis (Yumoto et al. 2002).

2.4.8.3 Other physiological and pathological roles

In addition to the apparent roles in the skeleton and immune system, TRACP may have a more widespread physiological function. It has been proposed to play a role (1) in iron metabolism, (2) as a growth and differentiation factor, (3) in lysosomal protein phosphorylation, and (4) in various pathological processes.

TRACP purified from pig uterus (uteroferrin) is a monomeric enzyme (Ketcham et al. 1985, Ling and Roberts 1993), and therefore considered as a TRACP 5a form. The enzyme has been suggested to function in iron transport from the mother to the developing fetus, and it is almost immediately degraded after losing its iron (Buhi et al. 1982). It has been suggested that the circulating enzyme may also release and recycle its iron before the enzyme is destroyed (Halleen et al. 1998b). Iron has been shown to directly regulate TRACP expression at the transcriptional level (Alcantara et al. 1994, Reddy et al. 1995), and an increased serum TRACP 5a level has been observed in patients with hemochromatosis, an inherited blood disorder that causes the body to retain excessive amounts of iron (Janckila et al. 2004).

A role for TRACP as a growth and differentiation factor in osteoblasts and adipocytes has been proposed. In some studies TRACP activity is detected in osteoblasts (Bianco et al. 1988, Bonucci et al. 2001, Perez-Amodio et al. 2005). The expression seems to be particularly obvious at the vicinity of resorbing osteoclasts, suggesting that osteoclasts somehow induce TRACP activity in osteoblasts. It has been suggested that TRACP secreted from osteoclasts would function as a regulator of osteoblast differentiation. However, some studies have demonstrated controversial observations. TRACP has been suggested to act as a coupling factor between osteoclasts and osteoblasts by interacting with TGF-β receptor interacting protein (TRIP-1) (Sheu et al. 2003). The interaction has been shown to induce a TGF-β-like differentiation process of osteoblasts increasing the activity and expression of ALP, collagen, osteoprotegerin and Runx2. In contrast, analysis of bone matrix production by osteoblasts isolated from TRACP knock-out mice showed an increased production of ALP and earlier mineralization, suggesting that TRACP would function as a negative regulator of osteoblast differentiation (Roberts et al. 2007a). In the course of breeding the TRACP overexpressing mice to a different genetic background, a sub-strain of the overexpressing mice presented with early onset obesity resulting in 20-40% increase in body weight (Lång et al. 2008). The obesity was suggested to be largely due to adipose tissue hyperplasia, and the mice exhibited signs of a low-grade inflammatory reaction in the adipose tissue without evidence of abnormal adipocyte lipolysis, lipogenesis or insulin sensitivity. These results suggested that specifically the monomeric TRACP 5a form, secreted by certain macrophages in the adipose tissue, causes the hyperplastic effects.

A recent study using both TRACP deficient mice and N1E-115 neuroblastoma cells overexpressing TRACP demonstrated that TRACP is essential for the removal of mannose-6-phosphate recognition marker from lysosomal proteins, and suggested that TRACP could play a role as a lysosomal phosphatase (Sun et al. 2008). In TRACP/LAP double knock-out mice macrophages of the liver, spleen, bone marrow and kidney exhibit excessive lysosomal storage (Suter et al. 2001). However, studies on fibroblasts from the double knock-out mice observed no effect in the dephosphorylation of endocytosed arylsulfatase A, suggesting that neither of the enzymes plays a role in dephosphorylation of lysosomal proteins.

Alterations in the lysosomal system accompany and may play a role in various disorders, including cancers. Studies on human breast cancer specimens have revealed a marked increase in the levels of mannose-6-phosphate containing glycoproteins in approximately one-third of tumors, which were associated with the more aggressive cases (Sleat et al. 1995). On the contrary, increased TRACP expression has been demonstrated in breast and ovarian cancers, and in melanoma (Honig et al. 2006), and the expression of TRACP in breast epithelial cells is increased when the tissue becomes malignant (Adams et al. 2007). Increase in OPN also correlates with enhanced malignancy, and OPN induces the migration of human mammary epithelial cells *in vitro*. It has been suggested that TRACP may also act on OPN in cancer cells and contribute to the development of metastases (Hayman 2008). A marked increase in serum TRACP concentrations has been observed in breast cancer patients with bone metastases (Chao et al. 2005b, Halleen et al. 2001, Mose et al. 2003), but also in patients with visceral metastases only (Honig et al. 2006).

2.4.9 Clinical use

2.4.9.1 Detection from serum

After its secretion, TRACP enters the circulation and forms a complex with α_2-macroglobulin and calcium (Brehme et al. 1999, Ylipahkala et al. 2003). Of the circulating enzyme, approximately 10% is estimated to be enzymatically active intact molecules, whereas 90% exists as inactive fragments (Halleen et al. 1996, Halleen et al. 1998a). Both the activity and quantity of the intact molecule can be measured from serum or plasma samples, and various immunoassays for these purposes have been developed. The first assays were kinetic assays measuring both TRACP 5a and 5b activity (Jalovaara et al. 1990, Lam et al. 1978b, Lau et al. 1987). The earlier immunoassays also measured both TRACP 5a and 5b activity (Echetebu et al. 1987, Lam et al. 1982, Miyazaki et al. 2003, Nakasato et al. 1999), or the quantity of both isoforms (Chamberlain et al. 1995, Cheung et al. 1995, Halleen et al. 1996, Halleen et al. 1998a, Kraenzlin et al. 1990). In normal physiological conditions, approximately 90% of the serum TRACP quantity is of 5a form, and therefore the amount of total TRACP provides a good estimate of the TRACP 5a quantity. Due to the higher specific activity of the 5b form, both isoforms represent approximately 50% of circulating TRACP activity, and therefore total TRACP activity reflects equally 5a and 5b activity. However, changes in either bone resorption or inflammatory conditions disturb this balance, and identifying the source of change by these assays is impossible.

More recently, assays specific for TRACP 5a and increased specificity for TRACP 5b have been developed (Halleen et al. 2000, Janckila et al. 2004, Nakanishi et al. 2000, Ohashi et al. 2007). 5a specificity is achieved by using a TRACP 5a specific monoclonal antibody that has enabled the development of immunoassays for TRACP 5a activity and quantity (Chao et al. 2005a). This far, TRACP 5b specific antibodies have not been developed, and therefore other approaches have been used to increase the 5b specificity. Three immunoassays with high specificity for TRACP 5b activity have been developed, one using a selective pH (Halleen et al. 2000) and the others using selective substrates (Janckila et al. 2004, Ohashi et al. 2007).

2.4.9.2 TRACP 5a: A marker of chronic inflammation?

TRACP 5a is the principal isoform secreted by macrophages and dendritic cells (Janckila et al. 2002). Elevated serum TRACP 5a levels have been observed in patients with hemochromatosis and rheumatoid arthritis (RA). Hemochromatosis patients have high levels of TRACP 5a activity (Janckila et al. 2004), whereas the TRACP 5a protein amount seems to be more clinically relevant in RA. Approximately one-third of RA patients have an elevated TRACP 5a protein amount (Chao et al. 2005a, Janckila et al. 2001a, Janckila et al. 2002). The elevated 5a protein shows a positive correlation with C-reactive protein, an acute-phase protein marker of inflammation and indicator of disease activity (Janckila et al. 2002), and with IgM rheumatoid factors (Janckila et al. 2008). During the course of the disease, approximately one-third of the RA patients develop nodular disease, which is often considered as a sign of more advanced and severe disease (Turesson et al. 2003). The 5a protein and IgM rheumatoid factors are shown to be significantly higher in RA patients with nodules than those without. The reason behind the lack of strong correlation between TRACP 5a activity and protein amount in RA is unclear (Janckila et al. 2008). TRACP 5a protein has also been

determined in patients with osteoarthritis, but the protein level does not seem not to be elevated (Janckila et al. 2002).

Studies suggest that TRACP 5a may be a clinically useful marker in chronic inflammatory diseases, and especially the TRACP 5a protein amount could be a prognostic marker for the degree of systemic inflammation and macrophage burden in RA. The TRACP 5a levels in RA show no correlation to TRACP 5b or other markers of bone metabolism, further demonstrating the different secretory origins of the 5a and 5b forms (Janckila et al. 2008).

2.4.9.3 TRACP 5b: A marker of osteoclast number

TRACP 5b is the principal isoform secreted by osteoclasts (Janckila et al. 2002). Increased serum TRACP 5b activity has been observed in the physiological conditions of growth and senescence, and in pathological conditions involving increased bone resorption such as osteoporosis, hyperparathyroidism, Gaucher's disease and some cancers (Capeller et al. 2003, Chamberlain et al. 1995). However, increased TRACP 5b activity has also been observed in patients affected by osteopetrosis (Alatalo et al. 2004), a disease resulting from impaired bone resorption, suggesting that the enzyme could be secreted from osteoclasts even if they are not resorbing.

Several studies suggest that secreted TRACP 5b is a marker of osteoclast number. Patients with autosomal recessive osteopetrosis and autosomal dominant osteopetrosis type II display impaired bone resorption despite a marked elevation of osteoclast number and serum TRACP 5b (Alatalo et al. 2004, Bollerslev et al. 1993, Del Fattore et al. 2006). Elevated TRACP 5b levels have also been detected in osteopetrotic rat strains (Alatalo et al. 2003a). Histological studies in patients with renal bone disease (Chu et al. 2003) and in orchidectomized rats (Alatalo et al. 2003b) demonstrate a strong correlation with the number of osteoclast and serum TRACP 5b. In addition, *in vitro* studies show that TRACP is released from the osteoclasts independently of whether the cells are seeded on plastic or bone slices (Alatalo et al. 2000, Karsdal et al. 2003). These studies indicate that TRACP 5b is secreted from both resorbing and non-resorbing osteoclasts, and therefore it reflects the number of the cells instead of their activity. However, conditions involving increased bone resorption are usually associated with an increase in osteoclast number, explaining the good performance of TRACP 5b as a marker of bone resorption in many clinical studies.

The most important clinical applications for bone resorption markers are monitoring treatment efficacy in postmenopausal osteoporosis and improving fracture risk assessment. TRACP 5b, reflecting osteoclast number, and the Cat K-generated degradation fragments of type I collagen (CTX and NTX) that reflect the functionality of osteoclasts, enable a more in-depth analysis of the changes in resorption. The use of TRACP 5b and markers of the collagen fragmentation products for monitoring antiresorptive treatment with alendronate, clodronate, HRT and raloxifene has been studied (Table 4). Inhibition of bone resorption may be achieved by a direct or indirect inhibition of osteoclastic resorption, by inhibition of osteoclastogenesis, or by induction of osteoclast apoptosis (Rodan and Martin 2000). Of the tested compounds, bisphosphonates may induce apoptosis in osteoclasts (Russell et al. 2007), whereas estrogen and SERMs act mainly by inhibiting osteoclastogenesis in addition to their functional effects (Rodan and Martin 2000). The compound AZD0530 is a highly

selective c-Src kinase inhibitor, which inhibits bone resorption without affecting the number of osteoclasts (Ple et al. 2004). Studies reveal differences in the marker responses according to the antiresorptive treatment. Bisphosphonate therapy decreases resorption by approximately 50-80% and reduces osteoclast number by approximately 20-40%, whereas the corresponding changes during HRT and raloxifene treatment are more similar. AZD0530 causes a dramatic decrease in resorption without major changes in osteoclast number. It has been suggested that assessment of the CTX to TRACP 5b ratio could improve the interpretation of changes in overall bone resorption during medical interventions (Rissanen et al. 2008).

Table 4. Serum TRACP 5b and CTX or NTX in clinical studies of different compounds and duration of treatment. Change of TRACP 5b and CTX/NTX is expressed in percentage of baseline level of the marker (modified from Henriksen et al. 2007).

Treatment		TRACP 5b	CTX/NTX	Reference
Alendronate	3 month	-40%	-64%	Nenonen et al. 2005
	6 month	-39%	-75%	Hannon et al. 2004
Clodronate	12 month	-18%	-51%	Tähtelä et al. 2005
HRT	6 month	-50%	-30%	Halleen et al. 2000
Raloxifene	3 month	-10%	-31%	Hansdottir et al. 2004
AZD0530	14 days	-11%	-88%	Hannon et al. 2005

Abbreviations: HRT, hormone replacement therapy; AZD0530, selective c-Src kinase inhibitor; TRACP 5b, tartrate-resistant acid phosphatase 5b; CTX, C-terminal crosslinked telopeptide of type I collagen; NTX, N-terminal crosslinked telopeptide of type I collagen.

The ability of TRACP 5b to predict fractures in elderly women was studied in a long-term study investigating incidence of fractures in elderly women followed for approximately 4.6 years. It was concluded that elevated levels of serum TRACP 5b were consistently associated with increased vertebral fracture risk (Gerdhem et al. 2004). The study has continued and results from a nine-year follow-up provide further support for the use of TRACP 5b in the assessment of fracture risk (Ivaska et al. 2007b).

TRACP 5b has some benefits compared to other commonly used bone turnover markers. Bone resorption exhibits a marked diurnal variation, with a peak during the night and a nadir in the morning (Schlemmer et al. 1992), which is also reflected in biochemical markers of bone resorption. However, the number of osteoclasts does not change dramatically during a 24-hour cycle (Simmons et al. 1988), and TRACP 5b has very little if any circadian rhythm (Halleen 2003, Hannon et al. 2004). In addition, the function of the kidneys has no effect on serum TRACP 5b levels, because the enzyme is cleared from the blood circulation through the liver (Saunders et al. 1985). In the case of hepatic failure, inactive fragments of TRACP accumulate in the serum, which do not affect the measurements of the active enzyme (Halleen et al. 2001).

3 AIMS OF THE STUDY

When the present study was initiated, an immunoassay with high specificity for osteoclastic TRACP 5b had been developed. However, the properties of the two isoforms of TRACP were only poorly understood, and the significance of TRACP 5a was unclear. Concomitant development of a TRACP 5a specific monoclonal antibody and immunoassays enabled us to broaden the knowledge of the differences between TRACP 5a and 5b. Further interest was focused in the clinical use of TRACP 5b. The following specific aims were set for this study.

1. To characterize the acid phosphatase and ROS generating activity of TRACP 5b.
2. To study the effects of antiresorptive treatment on TRACP 5b secretion.
3. To apply TRACP 5b and other bone markers to study changes in bone physiology during skeletal growth.

4 MATERIALS AND METHODS

4.1 Human recombinant TRACP (I)

4.1.1 Production and purification

Human recombinant TRACP (rTRACP) was produced in *Spodoptera frugiperda 9* (*Sf9*, clone ATCC) cells using a baculovirus construct containing human TRACP cDNA (Marshall et al. 1997). *Sf9* cells were grown as shaker cultures in Sf900II-medium (GibcoBRL, Life Technologies) supplemented with 4% fetal calf serum and 100 U/ml of penicillin. Cells were infected with the virus at multiplicity of infection 3, when cell density was approximately 2×10^6 cells/ml. Four days after infection the culture medium was collected and centrifuged at 2000 rcf for 5 minutes at RT. Cells were lysed with ice-cold lysis buffer (10 mM Tris pH 8.0, 130 mM NaCl, 1% Triton X-100, 1 mM PMSF) for 15 minutes at 4°C with gentle mixing and centrifuged at 40000 rcf for 45 minutes at 4°C.

Human rTRACP was purified from a pooled culture medium and cell lysate. 50 mM Hepes and 1 mM PMSF were added into the pool and the pH was adjusted to 8.0. The pool was then purified with a cation exchange chromatography column (CM Sepharose, Amersham Pharmacia Biotech AB, Uppsala, Sweden) equilibrated with 50 mM Hepes, pH 8.0, followed by elution of rTRACP from the column using a linear salt gradient (0-1.0 M NaCl in 50 mM Hepes, pH 8.0). Fractions containing phosphatase activity were pooled and concentrated with Centriprep® YM-10 concentrators (Amicon bioseparations, Millipore, Bedford, USA) according to the manufacturer's instructions in the presence of 0.05% Triton X-100. The sample buffer was then changed to 0.1 M Sodium Acetate buffer, pH 5.0, containing 0.3 M NaCl using commercial PD10 column (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Finally, rTRACP was purified with gel filtration chromatography (Sephacryl S-200 and S-100 columns connected together, 1.6 × 75 cm, Amersham Pharmacia Biotech AB, Uppsala, Sweden). The columns were equilibrated with 0.1 M Sodium Acetate, pH 5.0, containing 0.3 M NaCl before applying a 3.5 ml sample into the column at a speed of 30 ml/h. Fractions of 1.0 ml were collected and analyzed for phosphatase activity. Fractions containing activity were concentrated using Centriprep® YM-10 concentrators as described previously. All purification steps were performed at 4°C. Triton X-100 was omitted from the final preparation before measuring the ROS-generating activity. Purified rTRACP was stored at -70°C.

4.1.2 Cleavage with trypsin

Human rTRACP was cleaved using agarose-bound trypsin (40 mU/µg TRACP) (Sigma-Aldrich, St.Louis, MO, USA) in 20 µl of incubation buffer (10 mM Tris-HCl, pH 7.0, containing 0.1% Triton X-100) at RT for 24 hours with shaking. After the reaction, trypsin-bound agarose was removed with centrifugation at 16100 rcf for 5 minutes at RT. For ROS generating activity measurements, the cleavage was performed in 20 mM potassium phosphate buffer, pH 7.0.

4.1.3 Protein analysis

Total protein amounts from different purification steps were measured with the Bradford method using bovine serum albumin as a standard protein.

Samples from different purification steps and cleavage experiments were analyzed using 12% acrylamide separating gels. Silver staining was performed as previously described (Wray et al. 1981). A rabbit polyclonal anti-TRACP antibody (Alatalo et al. 2000) or a mouse monoclonal anti-TRACP 5a antibody Ab220 (Janckila et al. 2005) were used as primary antibodies in Western blotting experiments. Horseradish peroxidase-conjugated goat α-rabbit or goat α-mouse antibodies (Bio-Rad Laboratories, Richmond, CA, USA) were used as secondary antibodies, and visualized using diaminobenzidine tetrahydrochloride (DAB, Sigma, St. Louis, USA).

4.1.4 Measurement of acid phosphatase activity

The acid phosphatase activity of rTRACP was measured by incubating the samples at 37°C in the presence of 8 mM 4-nitrophenyl phosphate (4-NPP, Roche Diagnostics GmbH, Mannheim, Germany) and 33 mM L(+)-tartrate in 0.1 M sodium acetate buffer, pH 4.8-6.6. The reactions were stopped by adding sodium hydroxide, after which the absorbance was read at 405 nm using a model 2 Victor instrument (PerkinElmer Life Sciences-Wallac Oy, Turku, Finland). The measurements were also performed in the presence of 200 mM β-mercaptoethanol or 10 mM ascorbic acid (Sigma, St. Louis, USA).

Kinetic parameters were measured at the pH-optimum of monomeric and trypsin cleaved rTRACP using different amounts of substrate (0.7, 1.5, 3.0 and 5.9 mM 4-NPP). Michaelis-Menten curves were drawn and K_m, k_{cat}, and k_{cat}/K_m were calculated using GraphPad Prism software and nonlinear regression analysis.

4.1.5 Measurement of ROS generating activity

The ROS generating activity of rTRACP was measured using a method whereby coumarin-3-carboxylic acid (3-CCA, 99%, Sigma-Aldrich Chemie GmbH, Steinheim, Germany), a non-fluorescent molecule, is converted by hydroxyl radicals to a fluorescent product 7-hydroxycoumarin-3-carboxylic acid (7-OHCCA) (Ylipahkala et al. 2003). The reaction was performed using 0.5 μg rTRACP in 40 mM potassium phosphate buffer, pH 4.0-8.0, containing 200 μM 3-CCA, 1 mM hydrogen peroxide and 200 μM ascorbic acid. Before the reaction, rTRACP was preincubated with 17 mM ascorbate for 5 minutes. The amount of 7-OHCCA formed was measured using a model 2 Victor instrument (PerkinElmer Life Sciences-Wallac Oy) with an excitation wavelength of 355 nm and an emission wavelength of 460 nm.

Kinetic parameters were measured at pH 7.0 using different amounts of substrate (1.0, 2.5, 5.0 and 10.0 mM hydrogen peroxide). Michaelis-Menten curves were drawn and K_m, k_{cat}, and k_{cat}/K_m were calculated using GraphPad Prism software and nonlinear regression analysis.

4.2 Human osteoclasts (unpublished, II)

4.2.1 Cell cultures

Human peripheral blood mononuclear cells were isolated using Ficoll-Paque Plus solution (Amersham Pharmacia Biotech, Uppsala, Sweden) and sedimented as previously described (Husheem et al. 2005). The cells were attached on devitalized bovine cortical bone slices for two hours. The non-attached cells were then washed away and the remaining cells on the bone slices were cultured for ten days in α-minimum essential medium (Gibco BRL, Paisley, U.K.) supplemented with 10% iFBS (Gibco BRL, Paisley, U.K.), 20 ng/mL M-CSF (R&D Systems, Abingdon, U.K.), 40 ng/mL RANKL (Peprotech, London, U.K.), 100 IU/mL penicillin and 100 µg/mL streptomycin (Gibco BRL, Paisley, U.K.).

Human osteoclast precursor cells (Poietics; Cambrex, East Rutherford, NJ, USA) were cultured on devitalized bovine bone slices (Nordic Bioscience, Herlev, Denmark) for seven (in osteoclast differentiation assay) or ten (in osteoclast activity assay) days in medium supplemented with 33 ng/ml M-CSF and 66 ng/ml RANKL (Cambrex). In the osteoclast differentiation assay osteoprotegerin (PeproTech EC, London, UK) was added at the beginning of the cell culture and alendronate (Merck Research Labs, San Diego, CA, USA) was coated onto bone slices before adding the cells. In the osteoclast activity assay, cysteine protease inhibitor E64 (Sigma-Aldrich) and Cat K inhibitor ORG-29762 (Organon Laboratories, Newhouse, Scotland) were added at day seven. Cells were fixed with 3% paraformaldehyde for 20 min and stained for TRACP activity (Leukocyte acid phosphatase kit; Sigma-Aldrich, St Louis, MO, USA) and Hoechst 33258 (Sigma-Aldrich).

4.2.2 Immunofuorescence staining

The cells were fixed with 3% paraformaldehyde for 20 min and permeabilized with PBS containing 0.1% Triton X-100, followed by blocking with 2% BSA-PBS. Monoclonal antibody 220 (Janckila et al. 2005) (diluted 1:500) and polyclonal rabbit anti-TRACP (Alatalo et al. 2000) (diluted 1:800) were used as primary antibodies. Alexa Fluor 647 goat anti-mouse immunoglobulin (Molecular Probes) and fluorescein isothiocyanate (FITC) conjugated anti-rabbit immunoglobulins were used as secondary antibodies. Tetramethyl-rhodamine isothiocyanate (TRITC) conjugated phalloidin (Jackson Immunoresearch Laboratories, USA) was used for visualization of actin. The samples were examined using a Leica TCS-SP confocal laser scanning microscope equipped with an Argon-Krypton laser (Leica Microsystems, Heidelberg GmbH, Heidelberg, Germany).

4.3 Study subjects (III, IV)

4.3.1 Postmenopausal women treated with alendronate

The first clinical study was a one-year double-blind placebo-controlled randomized intervention trial in postmenopausal women and consisted of two experimental groups (Nenonen et al. 2005, Uusi-Rasi et al. 2003). One group received 5 mg alendronate (Fosamax; Merck & Co., Rahway, NJ, USA) daily (n = 70) and the other received placebo pills (n = 67). All subjects in both groups received a daily supplement of calcium carbonate (630 mg) and vitamin D (200 IU = 5 μg; Citracal + D; Mission Pharmacal, San Antonio, TX, USA). All subjects provided written informed consent before the study, and the study protocol was approved by a local research committee and the independent Medical Ethics Committee at Tampere University Hospital.

4.3.2 Peripubertal girls

The second clinical study consisted of 173 healthy Caucasian girls aged 10-17 years, who participated in a long-term health study (Lehtonen-Veromaa et al. 2000). After 6 years 33 girls were lost to follow-up, which diminished the study group to 140 subjects. All subjects were volunteers and were informed about the study before accepting the invitation to participate. Written informed consent was obtained from each subject and, if a minor, from her parent or guardian. The study was approved by the Ethics Review Committee of the Hospital District of Southwestern Finland and it was carried out in accordance with the Declaration of Helsinki.

4.4 Immunoassays (II, III, IV)

All blood samples from the study subjects were obtained after overnight fasting. Serum was separated by centrifugation and stored at -70°C until the analyses. In the study of peripubertal girls (IV), the samples were collected at the same time in the morning and at the same early follicular phase of the menstrual cycle. To avoid seasonal variation, the samples were collected during winter.

4.4.1 TRACP immunoassays

4.4.1.1 TRACP 5b activity with a selective pH (Act5b)

Act5b is an in-house immunoassay that uses a monoclonal anti-TRACP antibody O1A to immobilize TRACP and 4-NPP as a substrate to measure bound activity at the selective reaction pH 6.1, where TRACP 5a is considerably less active than TRACP 5b (Halleen et al. 2000). Briefly, anti-mouse IgG-coated microtiter wells (PerkinElmer Life Sciences, Wallac Oy, Finland) were coated with O1A. Standards were prepared by serial dilution of recombinant human TRACP equivalent to 0.32 to 10 U/L. Standards and samples were added and incubated for one hour at RT. Bound TRACP activity was measured using 4-NPP as a substrate in sodium acetate buffer, pH 6.1. The substrate solution was incubated for one hour at 37°C and the reaction was stopped by the addition of NaOH. Absorbance was read at 405 nm (model 2 Victor instrument, PerkinElmer Life Sciences, Wallac Oy, Finland) and bound

TRACP activity was expressed as U/L. A commercial version of the same immunoassay (BoneTRAP® assay, IDS Ltd, Boldon, UK) was used in the human osteoclast study (II).

4.4.1.2 TRACP 5b activity with a selective substrate (ASBI)

The ASBI immunoassay uses a monoclonal anti-TRACP antibody 14G6 for TRACP immobilization and a fluorogenic naphthol ASBI phosphate (N-ASBI-P) as a selective substrate for TRACP 5b at pH 6.3 (Janckila et al. 2004). Briefly, black opaque wells, pre-coated with neutravidin (Pierce Chemical Co.) were coated with biotinylated 14G6. Serum samples were diluted 1:5 and incubated in the wells for 16 h at 4°C. Bound TRACP activity was measured using N-ASBI-P as the substrate in MES buffer, pH 6.3, containing sodium tartrate, ethylene glycol monomethyl ether (EGME) and Nonidet P-40 (NP-40). Standards included 0, 2, 5, 10, 15 and 20 IU (µmol substrate hydrolyzed/min/l) of N-ASBI. The reaction was carried out for 30 min at 37°C, after which the reaction was stopped by addition of KOH. Fluorescence was measured using an 400/10 nm excitation filter and a 485/40 nm emission filter (BioTek Instruments, Winooski, VT). The results were expressed as IU TRACP activity.

4.4.1.3 TRACP 5 activity (Act5)

The Act5 immunoassay (Human TRAP5 Assay, BioVendor, Brno, Czech Republic) uses a TRACP antibody not identified by the manufacturer of the assay, and 4-NPP as the substrate at pH 5.5. The measurements were performed according to the manufacturer's instructions.

4.4.1.4 TRACP 5 protein (Prot5)

The Prot5 two-site immunoassay uses 14G6 as a capture antibody, and a peroxidase-conjugated monoclonal anti-TRACP antibody J1B (J1B-HRP) for detection (Nakasato et al. 1999). Briefly, pre-blocked avidin-coated wells were coated with 14G6-biotin. Samples and standards were added and incubated overnight at 4°C. Standards were made by serial dilution of partially purified serum TRACP 5a equivalent to 0.08 to 5 µg/L. To detect the bound TRACP protein, JIB-HRP was added and incubated for one hour at RT. After washing, the HRP activity was measured using ortho-phenylenediamine and H_2O_2 in citrate phosphate buffer, pH 5.0. The substrate was incubated for 15 min at RT, and the reaction was stopped by the addition of H_2SO_4. The absorbance was read at 490 nm, and bound TRACP protein was expressed as µg/L.

4.4.1.5 TRACP 5a activity (Act5a)

The Act5a immunoassay uses a monoclonal anti-TRACP 5a antibody 220 to immobilize TRACP 5a. The bound activity is measured at pH 5.8 (Chao et al. 2005a). Briefly, neutravidin-coated wells (Pierce Chemical Co.) were coated with biotinylated antibody 220. Serum samples were diluted 1:10 and added to the wells. The samples were incubated overnight at 4°C. Bound TRACP activity was measured using 4-NPP

as the substrate in sodium acetate buffer, pH 5.8. The substrate solution was incubated for one hour at 37°C and the reaction was stopped by the addition of NaOH. Standards were constructed from serial dilutions of 4-nitrophenolate equivalent to 0.16 to 10 U/L. Absorbance was read at 405 nm (BioRad Microplate 550 Reader), and bound TRACP activity was expressed as U/L.

4.4.1.6 TRACP 5a protein (Prot5a)

The Prot5a assay uses monoclonal antibody 220 to immobilize TRACP 5a. The bound protein is detected with J1B-HRP (Chao et al. 2005a). Briefly, neutravidin-coated wells (Pierce Chemical Co.) were coated with biotinylated antibody 220. Serum samples were diluted 1:10 and added to the wells. Samples and standards were incubated overnight at +4°C. Standards were made by serial dilution of partially purified serum TRACP 5a equivalent to 0.08 to 5 µg/L. To detect the bound TRACP 5a protein, JIB-HRP was added and incubated for one hour at RT. A substrate solution, consisting of ortho-phenylenediamine and H_2O_2 in a citrate phosphate buffer, pH 5.0, was incubated in the wells for 15 min at RT, after which the reaction was stopped by addition of H_2SO_4. Absorbance was read at 490 nm (BioRad Microplate 550 Reader), and bound TRACP 5a protein was expressed as µg/L.

4.4.2 Other bone markers

Serum CTX was measured using two commercial assays. In the human osteoclast study (II) and in the study of peripubertal girls (IV), CTX was measured by the CrossLaps® method of Nordic Bioscience (Herlev, Denmark). In the alendronate study (III), the measurements were performed with the method of Roche Diagnostics (Mannheim, Germany). Serum PINP was measured by a radioimmunoassay (Orion Diagnostica, Espoo, Finland). Serum BALP was measured by an immunoassay (Metra Biosystems, Mountain View, CA). Serum osteocalcin (OC) was measured by radioimmunoassay (CIS-Bio International, Gifsur-Yvette, France). All measurements were performed according to the manufacturers' instructions.

4.5 Bone mineral density measurements (III, IV)

In the alendronate study (III), lumbar spine BMD was measured with dual energy X-ray absorptiometry (DXA) (XR; Norland, Fort Atkinson, WI, USA) at baseline and at 12 months according to standard procedures (Sievänen et al. 1996). In the study of peripubertal girls (IV), the BMD of the femoral neck (FnBMD) and lumbar spine (L2-L4, LsBMD) were measured by DXA (QDR 4500C, Hologic, Inc., Waltham, MA) at baseline and at 2 and 6 years according to standard procedures (Lehtonen-Veromaa et al. 2000).

4.6 Statistical analysis (I-IV)

Statistical analysis was carried out using SPSS 15.0 for Windows (SPSS, Chicago, IL, USA), Origin 6.1 (OriginLab Corporation, Northampton, MA, USA) and Statistica 7.1 (Statsoft Inc, Tulsa, OK, USA). The Shapiro-Wilk test for gaussian distribution was applied for all markers, and in the case of nongaussian distributions, statistical calculations were done after logarithmic transformation. Pearson correlation was used for correlation analysis. Values of $p<0.05$ were considered significant.

In the alendronate study (III), the dependent and independent t-test was used to test the significance of marker changes at 3 months. Analytical variability (CVa) was determined as the mean CV of all duplicated measurements performed. Biological variability (CVi) was determined as the mean CV of the changes observed at 3 months compared with baseline in the placebo group. One-sided least significant change (LSC) at $p<0.05$ was determined for each method based on the analytical and biological variability using the equation: $LSC = 2.33 \times \sqrt{(CVa^2 + CVi^2)}$ (Hannon et al. 2004). Subjects that showed a decrease of more than LSC at 3 months were considered responders to the treatment. Specificity was determined as the percentage of non-responders in the placebo group, and sensitivity as the percentage of responders in the alendronate group. Area under the curve (AUC) values were determined from receiver operating characteristic (ROC) curves. Signal-to-noise ratio was determined by dividing the change of each marker in the alendronate group at 3 months with the marker's analytical and biological variability [$\sqrt{(CVa^2 + CVi^2)}$].

In the study of peripubertal girls (IV), standardized regression coefficients ($Beta_{std}$) were used for correlation analysis. The values of bone markers in different age groups were compared to the values of the highest age group using Kruskal-Wallis and Mann-Whitney U-tests.

5 RESULTS

5.1 Enzymatic properties of TRACP (I)

Human rTRACP was purified from pooled culture medium and cell lysate using cation exchange chromatography (CM Sepharose column) and gel filtration (Sephacryl S-200 & S-100 columns). The enzyme was purified 26-fold after the CM Sepharose column and 84-fold after the gel filtration. Approximately 8 mg of human rTRACP was purified from a one liter culture, with 87% recovery of rTRACP activity. The purified protein was purple colored and had a specific activity of 92.1 U/mg. SDS-PAGE analysis of the purified enzyme revealed a single protein band of 35 kD.

5.1.1 Effects of cleavage and reduction

Agarose-bound trypsin was used to cleave the rTRACP. After cleavage, SDS-PAGE analysis was performed in the presence of β-mercaptoethanol. Trypsin cleaved most of the rTRACP into two subunits, sized approximately 21 and 16 kD. The effects of cleavage on the antigenic properties of rTRACP were studied with Western blot analysis using two antibodies, a polyclonal rabbit anti-TRACP antibody and a monoclonal antibody Ab220, which is specific for the non-cleaved protease-sensitive loop peptide (Janckila et al. 2005). The polyclonal antibody recognized both the intact rTRACP and the cleaved subunits. Ab220 recognized only the intact rTRACP but not the cleaved subunits. SDS-PAGE analysis of rTRACP after trypsin cleavage revealed that the subunits were held together by a disulfide bond that was reduced with β-mercaptoethanol, but not with ascorbate.

The K_m of the phosphatase activity was only slightly affected by cleavage, but it was doubled by β-mercaptoethanol and decreased 50% by ascorbate. Both trypsin cleavage and reduction by β-mercaptoethanol increased the k_{cat} value 2-fold, and reduction by ascorbate increased it 3-fold. The combined action of trypsin cleavage and the reduction by β-mercaptoethanol increased the k_{cat} 14-fold, and the combined action of trypsin cleavage and reduction by ascorbate increased it 6-fold. As a result, k_{cat}/K_m was doubled by trypsin cleavage, increased 6-7-fold by reduction by ascorbate, and increased 9-fold by the combined action of trypsin cleavage and reduction by β-mercaptoethanol. The pH-optimum of the phosphatase activity was not affected by β-mercaptoethanol, but reduction by ascorbate and trypsin cleavage changed it from 5.4-5.6 to 6.2-6.4.

Ascorbate was essential for the ROS generating activity, and therefore all ROS-generating activity measurements were performed in the presence of ascorbate. The effects of β-mercaptoethanol could not be studied because of its action as a hydroxyl radical scavenger (Stanton et al. 1993). Trypsin cleavage decreased K_m slightly and increased k_{cat}, resulting as a 2.5-fold increase in k_{cat}/K_m in the presence of ascorbate. The pH-optimum of the ROS generating activity was 7.0, and it was not affected by trypsin cleavage.

5.2 TRACP in human osteoclast cultures (unpublished, II)

5.2.1 Intracellular localization

Osteoclasts differentiated from human peripheral blood were stained with the TRACP 5a specific monoclonal antibody 220 and with the polyclonal rabbit anti-TRACP antibody detecting both 5a and 5b forms. As a consequence of Ab220 incubation preceding the polyclonal antibody incubation, the polyclonal antibody was no longer able to detect the 5a form. However, after heat induced epitope retrieval, the polyclonal antibody was also able to detect the 5a form, and demonstrated a strong co-localization with Ab220 (data not shown). TRACP 5a was predominantly located at the center and perinuclear Golgi area, whereas TRACP 5b was detected closer to the ruffled border and in larger vesicles throughout the cell (Figure 8).

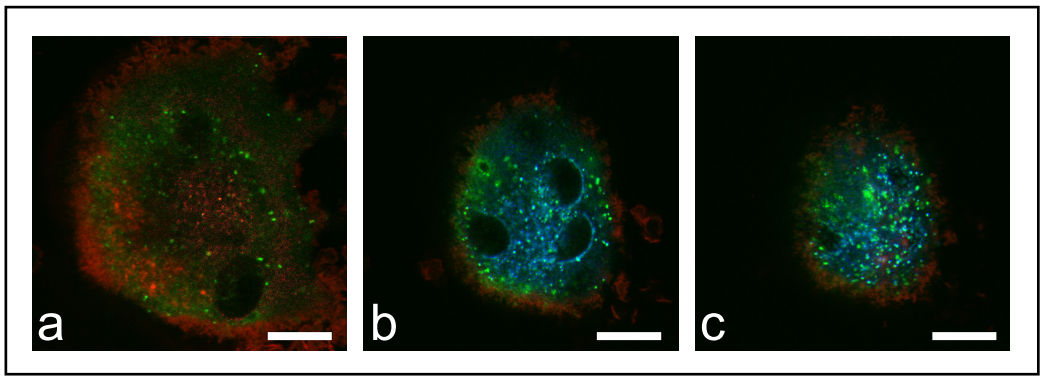

Figure 8. Intracellular localization of TRACP 5a (blue) and TRACP 5b (green) in a human osteoclast. (a) Localization at the lower level of the cell, (b) at the middle section, and (c) at the level of FSD. Actin (red) is visualized using TRITC-labeled phalloidin (Bars, 10 μm).

5.2.2 Effects of antiresorptive compounds

Two compounds, OPG and alendronate, were used for studying the association of osteoclast number, apoptosis and secretion of TRACP 5b. OPG significantly decreased osteoclast number and secretion of TRACP 5b in a concentration-dependent manner without affecting osteoclast apoptosis. However, the highest doses decreased the number of osteoclasts to close to zero while the medium TRACP 5b values remained at approximately 40% from the baseline level. Alendronate decreased osteoclast number and secretion of TRACP 5b, and at the same time increased osteoclast apoptosis in a concentration-dependent manner. With both compounds, the TRACP 5b values obtained showed a strong correlation with the microscopically counted number of osteoclasts.

The effects of the cysteine protease inhibitor E64 and Cat K inhibitor ORG-29762 in osteoclast activity were assessed for the development of an improved method for testing antiresorptive compounds. The compounds were added at day 7, after the completion of the osteoclast differentiation period. Both E64 and ORG-29762 inhibited the release of CTX into the culture medium. When the CTX values obtained after the

resorption period (at day 10) were normalized by the TRACP 5b values obtained at day 7, the resulting index CTX/TRACP 5b showed a concentration-dependent decrease in both experiments.

5.3 TRACP in monitoring alendronate treatment (III)

The baseline characteristics of the placebo and alendronate group showed no statistically significant differences between the groups (Nenonen et al. 2005). TRACP values decreased in both the alendronate and placebo groups after 3 months of treatment with Act5b (42% and 13%, respectively), ASBI (33% and 9%), Act5 (27% and 9%) and Prot5 (7% and 6%) assays, and the decrease was greater in the alendronate group ($p<0.001$) with Act5b, ASBI and Act5 assays. Significant changes were not observed with the Act5a and Prot5a assays in the alendronate group. The changes of CTX, PINP and other bone markers have been published earlier (Nenonen et al. 2005). The decrease was greater in the alendronate group for all other bone markers ($p<0.001$), the difference being greatest for CTX (64% in alendronate group and 22% in placebo group) and PINP (54% in alendronate group and 12% in placebo group).

The TRACP assays studied were compared for their clinical performance for monitoring alendronate treatment. CVa, CVi, LSC, specificity, sensitivity, signal-to-noise ratio and the AUC values of each assay were determined. The highest sensitivities were observed for Act5b (80.0%), ASBI (60.0%) and Act5 (60.0%), the highest AUC values for Act5b (0.93), Act5 (0.86) and ASBI (0.84), and the highest signal-to-noise ratios for Act5b (3.19), Act5 (2.63) and ASBI (2.51). Prot5, Act5a and Prot5a assays showed poor sensitivities, AUC values and signal-to-noise ratios. CTX and PINP had similar clinical performance as that of the Act5b method (Nenonen et al. 2005). For CTX, the sensitivity was 78.7%, the AUC value was 0.87, and the signal-to-noise ratio was 2.82. The corresponding values of PINP were 73.3%, 0.86 and 2.95.

A substantial decrease was also observed with change of the ratio of CTX to TRACP 5b (Table 5), which was determined using the results obtained with the TRACP assay possessing the highest clinical performance (Act5b).

Table 5. Changes of CTX (ΔCTX) and TRACP 5b (ΔTRACP 5b), and the ratio of CTX to TRACP 5b (ΔCTX/TRACP 5b) in response to three months alendronate treatment.

Group	ΔCTX	ΔTRACP 5b	ΔCTX/TRACP 5b
ALN	64 % ***	-42% ***	-42% ***
Placebo	22 % ***	-13% ***	-12% ***

***p<0.001 compared to baseline.

The Act5b, ASBI and Act5 assays correlated strongly with each other. Of these, the ASBI assay showed the weakest correlation with the Prot5, Act5a and Prot5a assays, indicating that it would have the lowest cross-reactivity with TRACP 5a. The Act5 assay showed a strong correlation with Prot5, Act5a and Prot5a assays, indicating a substantial cross-reactivity with TRACP 5a. The Prot5, Act5a and Prot5a assays

correlated strongly with each other. The strength of association between the different TRACP assays with CTX, PINP and BMD at baseline was analyzed. Act5b, ASBI and Act5 assays correlated with CTX and PINP. The Act5b assay was the only TRACP assay that correlated with BMD. The Prot5, Act5a and Prot5a assays did not correlate with CTX or PINP, with the exception that the Prot5 assay showed a weak correlation with CTX.

Similar to the baseline correlation analyses, changes of serum TRACP values in response to alendronate treatment observed with the Act5b, ASBI and Act5 assays correlated with each other. The changes in serum TRACP values observed with the Prot5 assay correlated with the changes in TRACP values obtained with all other TRACP assays, with the exception of the Act5a assay, for which a significant correlation was not observed. Changes in serum TRACP values observed with the Prot5a and Act5a assays showed no correlation with the changes in values obtained with any other TRACP assays. Similar to the analyses at baseline, the changes in serum TRACP values obtained using the Act5b, ASBI and Act5 assays correlated with concordant changes in CTX and PINP. The changes in serum TRACP values observed with the Act5b assay showed the strongest correlations with the changes in CTX and PINP. Act5b was the only assay whose changes correlated with the changes in BMD. The changes in serum TRACP values obtained using the Prot5, Act5a and Prot5a assays did not correlate with the changes in CTX or PINP, with the exception that a strong inverse correlation was observed between Prot5a and PINP.

5.4 Bone markers in peripubertal girls (IV)

The study subjects, consisting of peripubertal girls aged 10-17 years, were divided into seven groups according the years of menarche: -2 to -1 years, -1 to 0 years, 0 to 1 years, 1 to 2 years, 2 to 3 years, 3 to 4 years, and 4 to 5 years. At baseline, 12 of the 173 girls used oral contraception with low-dose estrogen (≤35μg). Serum CTX, TRACP 5b, PINP, BALP and OC were measured at baseline and after 2 years. All bone turnover markers showed a strong positive correlation with each other and a negative correlation with BMD at baseline. Both the baseline values of the bone markers and the mean of the bone marker values obtained at baseline and 2 years correlated with the changes of BMD during 6 years. Stronger correlations were observed with LsBMD than with FnBMD. The correlation coefficients were slightly higher with the mean bone marker values. The correlation coefficients were the same when the use of oral contraception during the 6 year period was excluded (n = 48, data not shown).

The levels of bone turnover markers in different age groups were determined. The highest levels of all bone turnover markers were observed before the age of menarche (10-13 years), and the levels decreased thereafter. The same marker levels and changes were observed when the use of oral contraception was excluded (data not shown). The ratio of CTX to TRACP 5b was lowest before the age of menarche, and increased thereafter. The ratio of PINP to BALP and PINP to OC was the highest, whereas the ratio of OC to BALP was lower before the age of menarche. The ratio of BALP to TRACP 5b did not change with increasing age.

6 DISCUSSION

6.1 Biological significance of TRACP 5a and 5b

The two isoforms of TRACP, 5a and 5b, are translated from the same gene and differ only after post-translational modifications, including processing of the repression loop and the carbohydrate content. As a result of these modifications, the isoforms possess different enzyme activity, pH optimum, and distinct preferences for synthetic substrates. A recent study elaborating the difference in the carbohydrate chain structure indicated that the differences between TRACP 5a and 5b consist not only of differences in sialic acid content, but also of differences in other sugar chain structures (Kawaguchi et al. 2008). Because the sugar chain is located in the vicinity of the repression loop, the difference could be involved in the enzyme maturation process, or it could lead to targeting of the enzyme to different subcellular locations. However, the major factor contributing to the differential properties of the isoforms seems to be proteolytical cleavage of the repression loop (Janckila et al. 2005). TRACPs purified from most human and rat tissues are obtained in a proteolytically cleaved two-subunit form, where the subunits are linked by a disulfide bond, whereas uteroferrin (TRACP in pig allantoic fluid) and recombinant TRACPs expressed in *Baculovirus* are purified as 5a-like single polypeptide. Various proteases such as Cat K, L and B, trypsin and papain are able to cleave the recombinant 5a-like form (Ljusberg et al. 1999, Ljusberg et al. 2005) and generate the more active 5b-like two-subunit form. The cleavage occurs at the repression loop, with a slight variance in the exact site and number of residues removed depending on the protease used. In osteoclasts, the most relevant protease to accomplish the cleavage is Cat K, whereas in macrophages Cat B or L are strong candidates (Lång and Andersson 2005). Moreover, Cat K has been shown to co-localize with TRACP in the transcytotic vesicles of osteoclasts (Vääräniemi et al. 2004), and an increased amount of uncleaved TRACP has been observed in the bones of Cat K knockout mice (Zenger et al. 2007).

In this study, human recombinant TRACP was expressed in *Sf9* cells using *Baculovirus* construct, and purified as monomeric TRACP 5a-like form. The recombinant TRACP produced was a heterogenous preparation of differentially glycosylated products, but it lacked sialic acid because the *Sf9* expression system does not have the biosynthetic capacity to synthesize and/or transfer sialic acid to glycoproteins (Altmann et al. 1999). The TRACP 5b-like form of the enzyme was achieved by proteolytic processing of the recombinant enzyme by trypsin, resulting in the two-subunit structure. The subunits were not recognized by an antibody specific for the loop structure of human TRACP 5a, indicating that part of the loop, most probably residues Asp156-Lys158 at the C terminal end of the loop, was removed. Consistent with other studies (Funhoff et al. 2001a, Ljusberg et al. 1999), an increase in acid phosphatase activity with concomitant change in pH optimum was observed. The enhanced activity resulting from cleavage is caused by an altered catalytic mechanism (Funhoff et al. 2005). In addition to the repression loop and pH, TRACP activity was shown to be regulated by the redox active iron and by the disulfide bond. Reduction of the redox-active iron activated the enzyme more efficiently than cleavage of the repression loop. However, combination of cleavage and reduction of either the redox-active iron or the disulfide bond resulted in more potent activation. Because the disulfide bond is located in the vicinity of the repression loop, linking the N and C terminal ends of the loop, it is reasonable to assume that activation of the cleaved form

as a consequence of the disulfide bond disruption is caused by a more open conformation of the active site. In the case of ROS generating activity, a neutral environment was observed to be optimal, consistent with the results achieved using different methods (Kaija et al. 2002). The 5b-like enzyme was substantially more active in producing ROS than the 5a-like form, but no difference was observed in the pH optimum of the two forms. Thus the more active 5b form may function as an acid phosphatase or as a ROS generator in different cellular compartments, and the activity predominating may be determined e.g. by the pH of the environment.

There is increasing evidence that bone and the immune system are functionally related, and several common regulatory molecules essential for the normal development and function of both systems have been identified (reviewed by Lorenzo et al. 2008, Rho et al. 2004). Furthermore, various studies have demonstrated a role for TRACP in bone and immune cells. Both the 5a and 5b form of TRACP are expressed in osteoclasts as well as in macrophages and dendritic cells, and in principle both the acid phosphatase and ROS generating activity of the enzyme may be utilized in these cells. OPN, also known as early T cell activation factor-1 (Eta-1), is considered to be the major substrate candidate for the acid phosphatase activity of TRACP, and the phosphorylation status of OPN has been suggested to regulate osteoclast attachment to bone (Andersson et al. 2003). The same molecule also interacts with macrophages to induce inflammatory responses. Localization of TRACP in the transcytotic vesicles of osteoclasts and in the antigen processing route of macrophages suggests a role for the enzyme in the processing of endocytosed material. For this function, the ROS generating activity of the enzyme may have an important role. Indeed, both macrophages from TRACP overexpressing mice and murine macrophage-like cell line RAW-264 overexpressing TRACP demonstrate an increased intracellular ROS production associated with increased bacterial killing (Räisänen et al. 2001, Räisänen et al. 2005). Consequently, a role for the ROS generating activity of TRACP in processing endocytosed material in macrophages and osteoclasts has been suggested (Halleen et al. 2003). In this concept, cleavage of TRACP by cysteine proteases, resulting in increased ROS production, may be important during both bone resorption and antigen presentation. Interestingly, it has been hypothesized that osteoclasts may be integral components of the immune and inflammatory responses (Baron 2004), having a specific role in attacking exposed bone surface recognized as a "foreign body" (Del Fattore et al. 2008). Presumably, osteoclasts would also need a machinery for processing the "foreign body", and intracellular processing of the resorption products could be an important mechanism in the prevention of autoimmune responses against internalized bone particles (Salo et al. 1997). Therefore, it could be possible that TRACP would have a role in antigen processing. This is supported by the observation of highly increased TRACP expression in precursors of osteoclasts as a consequence of exogenous DNA and RNA molecule introduction (Muhonen et al. 2007).

Human osteoclast cultures were used for studying intracellular TRACP 5a and 5b and secretion of TRACP 5b. Consistent with the activity measurements from cell lysates (Janckila et al. 2005), both TRACP 5a and 5b were detected by immunostaining in osteoclasts. The 5a form was predominantly located at the center and the perinuclear area of the cell, corresponding to the Golgi compartment, whereas the 5b form was detected in larger vesicles throughout the cell, and most likely within the transcytotic vesicles. Thus, osteoclastic 5a may represent a newly synthesized latent proenzyme,

which is later processed to an active, functional and secreted 5b form. Addition of osteoprotegerin or alendronate into the cultures resulted in a decrease of both osteoclast number and secretion of TRACP 5b, and a strong correlation between the two parameters. In the case of alendronate, concomitant and concentration-dependent increase in apoptosis was observed, indicating that the decreased number of osteoclasts is caused at least partly by induction of apoptosis. The results also demonstrate that TRACP 5b is not released from the apoptotic osteoclasts as an active enzyme, and it can be used as a marker of the number of living osteoclasts. The use of TRACP 5b as a marker of mature osteoclasts is supported by earlier studies, demonstrating that active TRACP 5b is not secreted from TRACP-positive osteoclast precursor cells (Alatalo et al. 2000). Despite the predominant secretion of the 5b form, a small but significant amount of the 5a form is also secreted by osteoclasts (Janckila et al. 2005), and hence the clinical significance of both serum TRACP 5a and 5b in response to commonly used alendronate treatment was assessed.

6.2 Serum TRACP 5a and 5b

The use of serum TRACP as a marker of bone turnover has been studied since the early 1970s. The earlier assays measuring either enzyme activity or quantity lacked the specificity to discriminate either TRACP 5a or TRACP 5b. The more recent TRACP 5a specific assays and assays with a high specificity for TRACP 5b have enabled the evaluation of the clinical usefulness of the isoforms. An increasing number of studies have demonstrated that serum TRACP 5a may be a clinically useful marker in chronic inflammatory diseases, whereas serum TRACP 5b is strongly correlated with the number of osteoclasts. In this study, we assessed the effects of alendronate on the secretion of TRACP 5a and TRACP 5b, and studied the age-dependent levels of TRACP 5b in peripubertal girls.

Bisphosphonates can affect osteoclast-mediated bone resorption in a variety of ways, including effects on osteoclast recruitment, differentiation and resorptive activity, and possibly effects on osteoclast morphology and apoptosis (reviewed by Russell et al. 2007). The effects also include impairment of intracellular vesicle transport, leading to an accumulation of TRACP containing vesicles (Alakangas et al. 2002). Alendronate treatment showed a marked decrease in values determined by three of the most TRACP 5b specific assays, Act5b, ASBI and Act5. The highest clinical performance was observed with the Act5b assay that uses a selective pH. The ASBI assay that uses a selective substrate demonstrated a total lack of correlation with TRACP 5a, concomitant with a high specificity of the assay. However, the overall clinical performance of the assay in this study was less desirable compared to the Act5b assay. A significant correlation with TRACP 5a was observed with the Act5 assay, suggesting that alterations in 5a level e.g. in inflammatory conditions are likely to decrease the clinical performance of the assay. TRACP 5a values showed no change in response to alendronate treatment, indicating that alendronate does not influence secretion of TRACP 5a from osteoclasts or from other cells. Similar results have been described with estrogen treatment (Halleen et al. 2002). In addition to the six assays used in this study, the clinical performance of a more recently released commercial immunoassay (MetraTRAP5b assay; Quidel, San Diego, USA) has been determined with the same panel of serum samples. This immunoassay uses two monoclonal antibodies, Trk49 and Trk62, to immobilize inactive TRACP fragments and active

TRACP, respectively. Bound TRACP activity is measured using TRACP 5b-selective CNPP as the substrate (Ohashi et al. 2007). The clinical performance of this assay was found to be equal with the Act5b assay (Ylipahkala et al. 2008).

Consistent with earlier findings (Rauchenzauner et al. 2007), the levels of TRACP 5b in peripubertal girls demonstrated similar age-dependent changes as was observed with other resorption and formation markers analyzed. The highest levels of TRACP 5b were observed just before the age of menarche, suggesting an increased overall number of osteoclasts at that time.

6.3 Interpretation of bone turnover markers

In addition to the traditional classification into markers of formation and markers of resorption, the biochemical markers of bone turnover can be divided into four categories: (1) markers that reflect bone resorption, (2) markers that reflect osteoclast number, (3) markers that reflect bone formation, and (4) markers which reflect increased osteoblast differentiation (Sørensen et al. 2007). In this study, the ratio of bone resorption to osteoclast number (CTX to TRACP 5b) was assessed in postmenopausal women treated with alendronate, and the levels and ratios of markers in the above-mentioned categories were determined in growing peripubertal girls. In children, bone metabolism is more complex than in adults, which makes the marker results more difficult to interpret. Basically, the marker levels can be similar in a child with high bone remodeling and low growth rate and in a normally growing child. However, in normal physiological conditions, a strong genetic impact is shown to control bone growth. It has been shown that sex, Tanner stage, whole-body mineral content, height velocity and whole-body mineral content accrual can significantly and independently explain 77-80% of the variability observed in bone turnover markers (Tuchman et al. 2008).

Monitoring treatment efficacy in postmenopausal osteoporosis is one of the most important clinical applications for bone resorption markers. Currently used antiresorptive osteoporosis drugs modulate bone resorption via alteration of both osteoclast number and function, and thus the combination of a marker of bone resorption and a marker of osteoclast number enables a more in-depth analysis of the changes in resorption. In addition, the presence of osteoclasts is essential for the shaping of the skeleton, but it may also be important for maintenance of the quality of adult skeleton. In normal adult bone turnover, bone formation is tightly coupled to bone resorption, and osteoclast function may contribute to the level of bone formation proceeding after bone resorption. It has been suggested that the resorptive activity of the cells does not control the coupling, but more likely osteoclasts themselves secrete or produce factor(s) contributing to induction of bone formation by osteoblasts, and the coupling would be controlled by the number of osteoclasts (Karsdal et al. 2007). Thus, in response to antiresorptive treatment, the decrease in osteoclast number can be associated with a secondary inhibition of bone formation, limiting the therapeutic window. Therefore, a compound that would attenuate the resorptive activity of osteoclasts without affecting the number of osteoclasts could result in continuous uncoupling of bone resorption and bone formation, and therefore be substantially more effective as a therapeutic agent for osteoporosis.

Increased levels of both TRACP 5b and markers of collagen fragmentation products are observed in postmenopausal women, indicating that both osteoclast number and resorption are elevated. Both CTX and TRACP 5b levels display a rapid decrease in response to alendronate treatment, suggesting that at least some of the antiresorptive effects are mediated via a decreased number of osteoclasts, due to either inhibition of osteoclast differentiation or their premature apoptotic death. Decreased levels of TRACP 5b could also be due to decreased secretion of the enzyme. A decrease was also observed in the ratio of CTX to TRACP 5b, suggesting a more prominent decrease in resorption than in the number of osteoclasts, and indicating decreased resorptive activity of the remaining cells. These results, together with the results obtained from the human osteoclast cultures, suggest that the assessment of the CTX to TRACP 5b ratio can improve the interpretation of the changes of overall bone resorption during medical interventions.

Differences in the ratio of CTX to TRACP 5b were also observed in peripubertal girls as a function of age and years from menarche. Interestingly, levels of the measured markers were highest at the time of menarche, whereas the ratio of CTX to TRACP 5b was lowest, suggesting the presence of a high number of osteoclasts with low activity. Further analysis of the formation markers also revealed differences in the ratios of PINP, BALP and OC. At the transcriptional level, PINP is an early synthesis product of proliferating osteoblasts, whereas BALP is expressed by differentiated osteoblasts during the maturation phase of collagen, and OC expression is associated with the later mineralization phase (Risteli and Risteli 1993, Stein et al. 1990). In this study, we observed that the ratio of PINP to BALP was highest before the age of menarche. A similar, or possibly even more prominent increase was observed in the ratio of PINP to OC, indicating increased collagen synthesis at that time. In contrast, the ratio of OC to BALP showed a different trend, being lower before the age of menarche and increasing thereafter. The different behavior of the ratio of BALP, PINP and OC in the different age groups of this study suggests that BALP would not reflect osteoblast activity in the same manner as PINP, but neither would it reflect matrix maturation in the same manner as OC. This leads to the suggestion that in analogy to TRACP 5b reflecting the number of osteoclasts, serum BALP could reflect the number of mature osteoblasts. However, it should be taken into account that some of the measured OC may be fragments derived from bone resorption, and further studies are needed to confirm this hypothesis.

7 CONCLUSIONS

Proteolytical cleavage of the TRACP 5a-like recombinant enzyme generates a TRACP 5b-like form with increased catalytic activity for both acid phosphatase and the ROS generating activity. Cleavage increases the sensitivity to reductants and changes the pH optimum of the acid phosphatase activity. However, reduction of the redox-active iron without cleavage is also an efficient way to activate the enzyme. The pH optimum of the ROS generating activity is not affected by cleavage.

Both TRACP 5a and 5b are present in osteoclasts. The 5a form is predominantly located at the perinuclear Golgi area, whereas the 5b form is detected in larger vesicles throughout the cell. Disturbance of the osteoclast function by alendronate decreases TRACP 5b secretion by 30-40%, demonstrating that alendronate decreases the number of osteoclasts. However, no change is observed in serum TRACP 5a levels, indicating that TRACP 5a is predominantly produced and secreted by other cells than osteoclasts. Consistently, human osteoclast cultures demonstrate that alendronate decreases the number of osteoclasts by inducing apoptosis, and TRACP 5b is not secreted as an active enzyme from the apoptotic osteoclasts.

Recently developed immunoassays with increased specificity for TRACP 5b and for total TRACP activity display similar responses to alendronate treatment, but differ in their clinical performance. The highest clinical performance and a significant inverse correlation of 3-month changes in TRACP values with 12-month changes in BMD are observed with the TRACP 5b assay using a selective pH.

Bone turnover markers predict changes in BMD in peripubertal girls. The marker levels are highest before the age of menarche (10-13 years), whereas the ratio of CTX to TRACP 5b is lowest, suggesting the presence of a high number of osteoclasts with low resorption activity. In contrast, the ratios of PINP to BALP and PINP to OC are highest before the age of menarche, indicating increased collagen synthesis at that time. The ratio of OC to BALP is lowest before the age of menarche and increases thereafter, suggesting that secretion of OC is associated with increased mineralization, whereas secreted BALP could reflect the number of mature osteoblasts. No change was observed in the ratio of BALP to TRACP 5b with increasing age. If BALP reflects the number of osteoblasts, the results would suggest that the number of osteoclasts changes similar to the number of osteoblasts throughout the growth period, whereas the bone formation and resorption activity of the cells change to opposite directions, leading to increased bone mass.

8 FUTURE ASPECTS

TRACP has been used as a histochemical marker of osteoclasts for more than 25 years, and it has become one of the best-known markers of osteoclasts and their precursors. The enzyme, once thought to be just a histochemical marker of osteoclasts, has gradually been uncovered as an enzyme with distinctive functions in both the skeleton and the immune system. Moreover, highly conserved structure of TRACP provides strong evidence for an important biological role for the enzyme. However, despite of intensive studies, the biological function and natural substrates of TRACP are still unclear. In osteoclasts, TRACP has been localized in cytoplasmic vesicles or vacuoles of various sizes and in small cytoplasmic granules. Moreover, TRACP has been co-localized with intracellular vesicles containing Cat K and endocytosed bone degradation products released from bone matrix during resorption. In some studies, TRACP has also been detected in the ruffled border area and in the resorption lacuna. These rather indefinite and diverse results suggest that TRACP may also be localized in some other, still unidentified vesicular compartments. Moreover, the ratio, localization and significance of TRACP 5a and 5b forms during different stages of osteoclast differentiation remain to be elaborated.

Serum TRACP 5b has established a strong status among markers of bone resorption, and is being increasingly studied. Its usability in assessing the number of osteoclasts clearly distinguishes it from the other markers of bone resorption. The highly specific TRACP 5b immunoassay utilized in this thesis has been demonstrated to be a reliable and specific method. However, new assays suitable for automated platforms would allow wider use of TRACP 5b measurements, thus creating a need for the development of TRACP 5b specific antibodies.

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