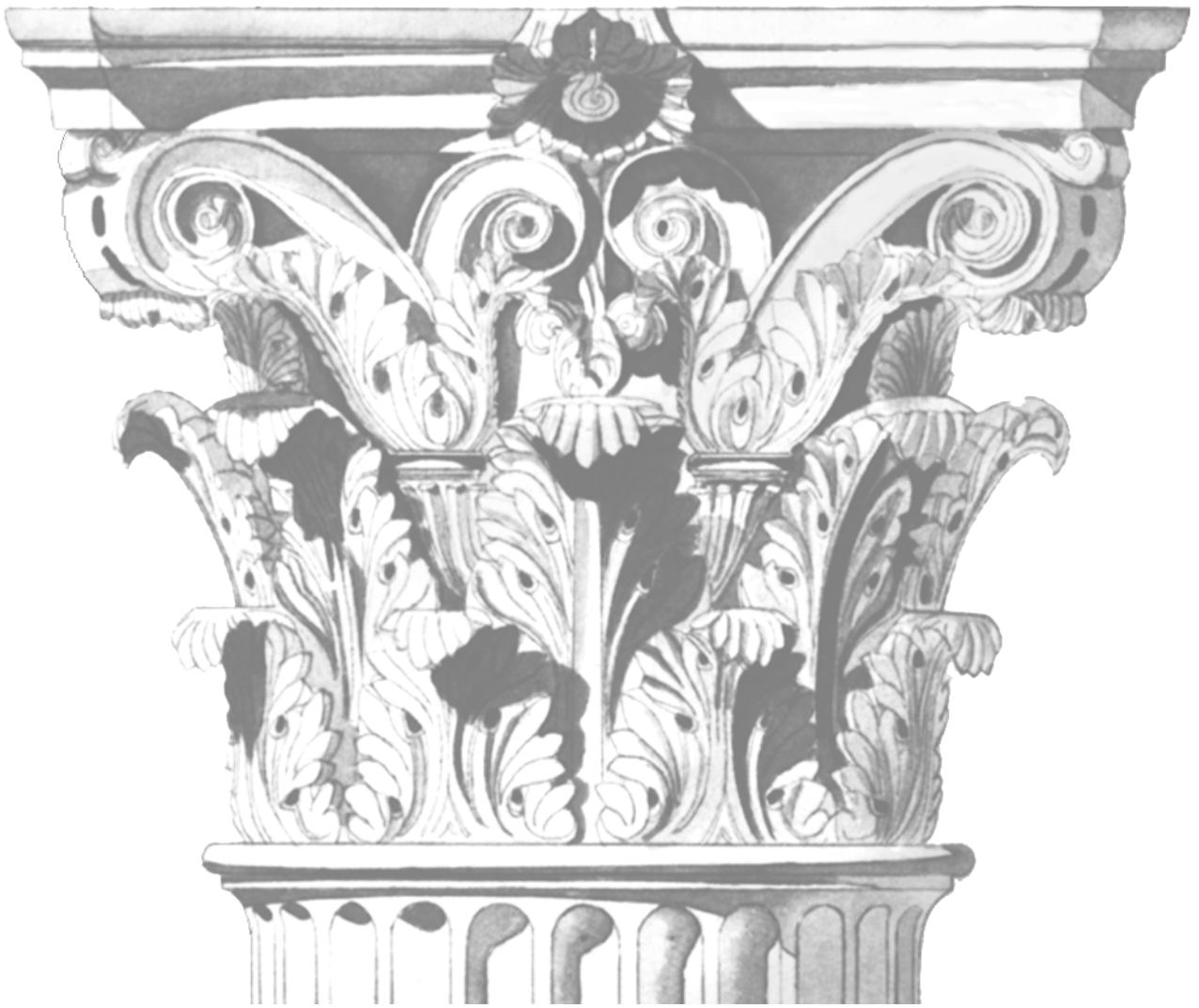
CARBOXYTERMINAL DEGRADATION PRODUCTS OF TYPE I COLLAGEN

MIRJA-LIISA SASSI

Department of Clinical Chemistry,
University of Oulu

OULU 2001



MIRJA-LIISA SASSI

CARBOXYTERMINAL DEGRADATION PRODUCTS OF TYPE I COLLAGEN

Academic Dissertation to be presented with the assent of the Faculty of Medicine, University of Oulu, for public discussion in the Auditorium 3 of the University Hospital of Oulu, on October 5th, 2001, at 12 noon.

OULUN YLIOPISTO, OULU 2001

Manuscript received 30 July 2001
Manuscript accepted 3 August 2001

Communicated by
Professor Jyrki Heino
Docent Pekka Hannonen

ISBN 951-42-6491-6 (URL: http://herkules.oulu.fi/isbn9514264916/)

ALSO AVAILABLE IN PRINTED FORMAT
ISBN 951-42-6490-8
ISSN 0355-3221 (URL: http://herkules.oulu.fi/issn03553221/)

OULU UNIVERSITY PRESS
OULU 2001

Sassi, Mirja-Liisa, Carboxyterminal degradation products of type I collagen
Department of Clinical Chemistry, University of Oulu, P.O.Box 5000, FIN-90014 University of Oulu, Finland
2001
Oulu, Finland
(Manuscript received 30 July 2001)

Abstract

The assay for the carboxyterminal telopeptide of type I collagen, ICTP, has been shown to be a reliable marker in many pathological conditions but insensitive to changes in physiological bone turnover. This has induced uncertainty and confusion regarding the role of ICTP assay in the study of collagen metabolism in bones. Especially, since another assay for the carboxyterminal telopeptide of type I collagen, serum CrossLaps ELISA, sensitively follows the changes in physiological bone turnover. To find out the reasons for the discrepancy we characterized the antigenic determinant of the ICTP assay by comparing human and bovine antigens after trypsin and chymotrypsin treatments. An assay for bovine ICTP was developed contemporarily with the present study. The epitope lies on the phenylalanine rich region of two telopeptide chains. We were able to show that the region is destroyed by cathepsin K, an osteoclastic enzyme responsible for physiological bone turnover, but not by several matrix metalloproteinases (MMPs), which are important collagen degrading enzymes in pathological conditions. Cathepsin K treatment had no effect on the CrossLaps assay. The CrossLaps assay is also able to measure the MMP-derived fragments, but usually their amount is so low in serum that it is masked by the cathepsin K-derived collagen degradation. The results explain the apparent discrepancy regarding the different behaviour of ICTP and CrossLaps assays in various conditions as also verified in our study with rheumatoid arthritis patients.

The ICTP assay was also found to measure only trivalently cross-linked forms of the carboxyterminal telopeptide which contains two telopeptide chains, and is therefore unable to react with divalently or histidinohydroxylysinonorleucine (HHL)-cross-linked forms of the carboxyterminal telopeptide. These forms can be measured with the SP4 (synthetic peptide 4) assay. We utilized this property in analyzing the skin samples of 18 breast cancer patients on both the irradiated and unirradiated side. The content of HHL was increased on the irradiated side, as were type I collagen synthesis and degradation.

In conclusion, there are two assays for two different degradation products of the trivalently cross-linked carboxyterminal telopeptide of type I collagen, ICTP and CrossLaps, the former measuring the MMP-derived and the latter the cathepsin K-derived collagen degradation.

Keywords: ICTP, CrossLaps, MMPs, Cathepsin K

To my dear Esko

Acknowledgements

This study was carried out at the exterior of the Department of Medical Biochemistry, University of Oulu, during the years 1995–1997 and at the Department of Clinical Chemistry and at the Laboratory of Oulu University Hospital during the years 1997–2001.

Professor Juha Risteli has been the supervisor of this thesis.

Docent Pekka Hannonen and professor Jyrki Heino revised this manuscript and Ms Anna Vuolteenaho revised the language of this manuscript.

Several collaborators have contributed to this study.

The laboratory technicians Kristiina Apajalahti, Ulla Pohjoisaho, Päivi Annala, Tiina Holappa, Sanna Sääskilahti and Liisa Kaarela have helped me doing most of the pipetting and keeping the laboratory all right. Dr. Aimo Heinämäki has been doing the heaviest and dirtiest parts of the work, e.g. cutting the bones.

The other members of “Risteli group” have been helping me with many technical and practical problems. I also want to thank Drs Tarja and Jukka Melkko and Dr Arja Jukkola for their kind words during the darkest days.

First of all I wish to thank Esko Puitti for his love. I wish to thank both of my wonderful families and the old and the many new friends I have got with Esko.

This thesis has been supported by Finnish Cancer Foundation, Oulu University Hospital and Technology Development Centre of Finland.

Deep in my soul I feel calling for doing research. I think that knowing the truth about things is valuable. But after these years I also understand that it is impossible to serve two Bosses. And the Boss I want to worship is not Science.

Oulu, August 2001 Mirja-Liisa Sassi

Abbreviations

ACP	aldol condensation product
AH	aldol histidine
AMC	aminomethylcoumarin
BiP	immunoglobulin heavy chain binding protein
BSA	bovine serum albumin
Cbz	benzyloxycarbonyl
cDNA	complement DNA
CLSPA	chromatographically purified collagenase
COL1A1	gene for the α1-chain of type I collagen
COL1A2	gene for the α2-chain of type I collagen
C(OOH)-terminal	carboxyterminal
CRP	C-reactive protein
DEAE	diethylaminoethyl
DHLN	dihyroxylysinonorleucine
Dpd	lysyl pyridinoline
D-Pyr	lysyl pyridinoline
ELISA	enzyme linked immunosorbent assay
GHYL	galactosyl hydroxylysine
Gly-X-Y	glycine-unknown amino acid-unknown amino acid
ECM	extracellular matrix
EDS	Ehlers-Danlos syndrome
EDTA	ethylenediamine tetra-acetate
ESR	erythrocyte sedimentation rate
HHL	histidinohydroxylysinonorleucine
HLN	hydroxylysinonorleucine
HP	hydroxylysyl pyridinoline
HPLC	high performance liquid chromatography
HRT	hormone replacement therapy
HSP47	heat shock protein 47
HYP	hydroxyproline
ICTP	carboxyterminal telopeptide of type I collagen

LN	lysinonorleucine
LP	lysyl pyridinoline
MALDI-TOF	matrix assisted laser desorption ionization - time of flight mass spectrometry
MMP	matrix metalloproteinase
MT-MMP	membrane type matrix metalloproteinase
MW	molecular weight
N-terminal	aminoterminal
Ntx	assay for the N-terminal telopeptide of type I collagen
OI	osteogenesis imperfecta
PA	pyridinoline analogue
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PDI	protein disulphide isomerase
PEG	polyethylene glycol
PICP	carboxyterminal propeptide of type I collagen
PIIINP	aminoterminal propeptide of type III collagen
PINP	aminoterminal propeptide of type I collagen
PML	polymorphonuclear leukocytes
PPI	peptidyl-prolyl cis-trans-isomerase
Pyr	hydroxylysyl pyridinoline
RER	rough endoplasmatic reticulum
RF	rheumatoid factor
SDS	sodium dodecyl sulphate
SF	synovial fluid
SP4	synthetic peptide 4; a linear peptide with amino acid sequence SAGFDFSFLPQPPQEKY
TIMP	tissue inhibitor of matrix metalloproteinase
TPCK	N-tosyl-$_L$-phenylalanine chloromethyl ketone
uPA	urokinase-type plasminogen activator
UV	ultraviolet

Contents

Acknowledgements
Abbreviations
Contents
1 Introduction 13
2 Review of the literature 14
2.1 Collagens in the animal kingdom 14
2.2 Type I collagen 15
2.3 Type III collagen 16
2.4 Type I collagen synthesis, posttranslational modifications and fibril formation . 17
2.5 Cross-linking of type I collagen 18
2.5.1 A short history of cross-link research 18
2.5.2 The divalent cross-links 19
2.5.3 The mature, trivalent cross-links 20
2.6 Degradation of type I collagen 21
2.6.1 Principles of the degrading enzymes 21
2.6.2 An aspartic proteinase, Cathepsin D 21
2.6.3 Cysteine proteases 22
2.6.3.1 Cathepsins B, L and S 22
2.6.3.2 Cathepsin K 22
2.6.3.3 Serine proteinases 24
2.6.4 Matrix metalloproteinases 25
2.7 Assessing the rate of type I collagen synthesis 28
2.8 Biochemical alternatives to measure collagen degradation 29
3 The aims of the study 31
4 Materials and methods 32
4.1 Isolation of type I collagen from human and bovine bone and from human uterine leiomyoma and human skin 32
4.2 Isolation of C-terminal telopeptides from the purified collagens 33
4.3 Chemical characterization of the purified peptides 33
4.4 Production of antibodies 34
4.5 Labelling of the peptides 34

4.6 Immunoassays 34
4.7 Patients (III, IV) 36
4.8 Animals (II) 36
4.9 Statistical analysis of data (III, IV) 37
4.10 Enzymatic degradation of the purified peptides 37
4.11 Analytical methods 37
5 Results 39
5.1 Purification and characterization of the carboxyterminal telopeptides of type I collagen (I, II, III, additional data) 39
5.2 Radioimmunoassay for bovine ICTP (II) 42
5.3 Characterization of the enzyme cleavage sites in ICTP (I) 42
5.4 Characterization and enzyme stability of the antigenic determinants of ICTP (I) ... 42
5.5 The specificity and enzyme stability of the CrossLaps assay – comparison with ICTP (IV, additional data) 43
5.6 Irradiation fibrosis (III) 46
5.7 Evaluation of ICTP and CrossLaps in rheumatoid arthritis and analysis of the serum and synovial fluid C-terminal degradation fragments (IV) 46
6 Discussion 48
7 Conclusions 52
References

1 Introduction

The degradation of the most common extracellular protein in mammalian tissues, type I collagen, is under strict physiological control but increases in several pathological conditions, e.g. osteoporosis, fibrosis, rheumatoid arthritis and various cancers. Thus, in addition to the established disease assessment tests, the markers for type I collagen degradation give an extra perspective on the disease process. Type I collagen degradation products can be followed in biological fluids with several assays developed during the last decade. These include the ICTP and CrossLaps assays and various assays measuring collagen cross-links, such as pyridinolines. All the above-mentioned assays measure differently processed fragments derived from the carboxyterminal telopeptide of type I collagen. Also an assay for the aminoterminal telopeptide of type I collagen, NTx, is available.

Although the origin of the antigens may be the same molecule, the assays behave differently in different clinical situations. The assay for the carboxyterminal telopeptide for type I collagen, ICTP, has been shown to be a reliable marker of pathological degradation of type I collagen in several diseases, e.g. in rheumatoid arthritis. However, in contrast to the CrossLaps assay, it is insensitive in reflecting physiological bone turnover. This study was designed to find out the reasons for the discrepancy.

Since type I collagen is found both in bones and soft tissues, it is of crucial importance to know e.g. which cell types and individual enzymes are involved in the degradation process in various conditions. The cleavage of collagen by different degrading enzymes, cathepsins and matrix metalloproteinases, produces fragments of carboxyterminal telopeptides of various sizes. In addition, the fine structure of the substrate, including the primary amino acid sequence as well as the secondary structure with several post-translational modifications of the carboxyterminal telopeptide (e.g. the nature of the cross-links and type and degree of glycation), determine whether the fragments are detected by a particular assay.

2 Review of the literature

2.1 Collagens in the animal kingdom

When the multicellular organisms evolved a material was needed between the cells to give the tissue shape, resistance to pressure, torsion and tension and to provide the structural frameworks to some specialized tissues, such as cartilage and bone. In addition, this material, extracellular matrix (ECM), is needed to keep the cells in contact with each other, in the development of tissues and for many other special functions. Even the very primitive multicellular animals have ECM, which is composed of proteoglycans, adhesive glycoproteins and collagens (Crazer 2000). The main types of collagen evolved about 800–900 million years ago, the same date as the fossil record of primitive Metazoa (Runnegar 1985). The collagen superfamily can roughly be divided in two divergent subfamilies, one of which includes the vertebrate interstitial collagen genes, and the other of which includes the invertebrate collagen genes and e.g. the vertebrate type IV and type IX collagen genes (Fields 1988). The evolution of genes of fibrillar collagens has been assumed to develop from a six Gly-X-Y-triplets coding early gene, since the collagen genes often have 54 base pairs long exons (Kivirikko & Myllylä 1983). However, type IV collagen genes appear not to be related to the 54-base-pair-coding unit (Sakurai *et al.* 1986). On the other hand, the 20 genetically distinct collagen types found so far in vertebrates may be grouped into seven or eight different families according to their structure and function (e.g. fibril-forming and basement membrane collagens) (Von der Mark 1999).

Collagens form triple helical conformations where polypeptide chains, so-called α chains, coil into a left-handed helix with about 18 amino acids per turn. The molecules have high hydroxyproline and hydroxylysine content and glycine as every third amino acid, prerequisite for triple helical folding. If an obligatory glycine is substituted by some bulkier amino acid in case of single base pair mutation, the propagation of the folding of the molecule is delayed, the residues N-terminally to the mutation site are overmodified and the stability of the triple helix is decreased as well as the secretion of procollagen and normal fibril formation (Engel & Prockop 1991). Glycine occupies the restricted space where the three helical α chains come together in the centre of the triple helix (Prockop *et al.* 1979). Each of the three chains therefore has the repeating structure Gly-X-Y, in

which X and Y can be any amino acid but are frequently the imino acids proline (about 100 of the X positions) and hydroxyproline (about 100 of the Y positions). Because both proline and hydroxyproline are rigid, cyclic amino acids, they limit rotation of the polypeptide backbone and thus contribute to the stability of the triple helix (Prockop *et al.* 1979). Hydroxyproline has an essential role in stabilizing the triple helix of collagen by hydrogen bonding between the hydroxyl group and water. Collagen polypeptides that lack hydroxyproline can fold into a triple-helical conformation at low temperatures, but the triple helix formed is not stable at mammalian body temperature (Prockop *et al.* 1979). The amount of Gly-Pro-Hyp sequences is the main, but not exclusive, factor for varying collagen thermostability ranging from Antarctic fish to animals living at very high temperatures near the thermal vent at the bottom of the ocean (Burjanadze 2000).

Hydroxylysine is important for the stability of the collagen fibres and serves as a site of attachment for the glycosyl units galactose and glycosylgalactose and collagen cross-linking. The exceptions from the triple helical model are the telopeptides at both heads of the molecule, the N-terminal telopeptide and C-terminal telopeptide, the least conserved parts of collagen molecules among species.

2.2 Type I collagen

When the term collagen is used, it usually means type I collagen, the most common of the collagens in vertebrates. It comprises up to 90% of the skeletons of the mammals and is also widespread all over the body: in addition to bones, it is found in skin, tendons, ligaments, cornea, intervertebral disks, dentine, arteries and granulation tissues as the main locations. Even cartilage, which mainly contains type II collagen, has been mentioned to contain some type I collagen (Wardale & Duance 1993). It is also widespread in the animal kingdom, from invertebrates (Exposito *et al.* 1992) to vertebrates. Type I collagen is also important in other respects: for example, it is used in the gelatin industry and in many biomaterials, and leather is, in fact, mostly composed of type I collagen. The importance of type I collagen for medical research is that it is involved in many human and animal diseases, including fibrosis, osteoporosis, cancer, atherosclerosis etc. In spite of or because of the fact that it is widely distributed in the body the different parts (degradation products) of type I collagen molecule are frequently utilized to monitor physiological changes in tissues as well as being used as diagnostic tools in various pathological conditions.

The structure of type I procollagen is shown in Figure 3. Similarly to other fibrillar collagens this molecule comprises three polypeptide chains (α-chains) which form a unique triple-helical structure. It is a heterotrimer of two α1(I) and one α2(I) chains. Among species, the α1(I) chain is more conserved than the α2(I) chain (Kimura 1983). Small amounts of homotrimer of three α1(I) chains have been found in embryonic tissues and in some individuals with osteogenesis imperfecta (OI). The bony fishes also have an α3(I) chain in their type I collagen. Type I collagen molecule contains an uninterrupted triple helix of approximately 300 nm in length and 1.5 nm in diameter flanked by short nonhelical telopeptides. The helical region is highly conserved among species (Chu *et al.*

1984). The telopeptides, which do not have a repeating Gly-X-Y structure and do not adopt a triple helical conformation, account for 2% of the molecule and are essential for fibril formation (Kadler *et al*. 1996). The telopeptides are the most immunogenic regions of type I molecule. The most carboxy-terminal part of the carboxy-terminal telopeptide of type I collagen α1-chain D-G-G-R-Y-Y is, however, highly conserved and activates polymorphonuclear leucocytes (Monboisse *et al*. 1990). The α2-chain, which does not have this sequence, also lacks this property. In addition, a specific property of the carboxyterminal telopeptide is that its α1-chain adopts a folded conformation with a sharp hairpin turn around residues 13 and 14 of the 25-residue telopeptide (Orgel *et al*. 2000). Both telopeptide regions take part in cross-link formation (see later).

Type I collagen molecules form D-periodic (D = 67 nm, the characteristic axial periodicity of collagen) cross-striated fibrils in the extracellular space, giving the tissues their mechanical strength and providing the major biomechanical scaffold for cell attachment and anchorage of macromolecules. Many macromolecules such as integrins, fibronectin, fibromodulin and decorin attach to type I collagen. Type I collagen also interacts with many cells, such as fibroblasts, and with platelets during blood clotting. In bones and dentin type I collagen is mineralized with hydroxyapatite crystals. The process is mediated by non-collagenous proteins after decorin molecules have been removed from the newly-synthesized collagen molecules (Hoshi *et al*. 1999). If the type I collagen gene is mutated, it leads to several forms of osteogenesis imperfecta (OI), characterized by brittle bones, Ehlers-Danlos syndrome (EDS), characterized by hypermobility of joints and abnormalities of skin, or Marfan syndrome, characterized by abnormalities in arteries (Kadler 1995).

2.3 Type III collagen

Type III collagen is the second most abundant collagen in human tissues and occurs particularly in tissues exhibiting elastic properties, such as skin, blood vessels and various internal organs. It is a homotrimer composed of three α1(III) chains and resembles other fibrillar collagens in its structure and function. Its elastic properties may be due to the disulphide bonds and the fact that there is no lysyl oxidase-dependent cross-link in the C-terminal end (Cheung *et al*. 1983). It is synthesized as procollagen similarly to type I collagen, but the N-terminal propeptide remains attached in the mature, fibrillar type III collagen more often than in type I. Mutations of type III collagen cause the most severe form of Ehlers-Danlos syndrome, EDS IV, which affect arteries, internal organs, joints and skin, and may cause sudden death when the large arteries rupture.

2.4 Type I collagen synthesis, posttranslational modifications and fibril formation

The main proportion of type I collagen in the mammalian body is produced by fibroblasts and osteoblasts, though many other cells are also able to synthesize it. The genes for two different α-chains, COL1A1 and COL1A2, lie in chromosomes 17 and 7, respectively.

Type I collagen is synthesized as procollagen molecules in the lumen of the rough endoplasmatic reticulum (RER), transported to the Golgi apparatus, and secreted into the extracellular space via secretory vesicles. However, a significant amount of newly synthesized collagen is degraded intracellularly in lysosomes (Ripley & Bienkowski 1997). The synthesis of collagen fibrils can be considered to occur in two stages: intracellular steps are required to assemble and secrete the procollagen molecule, and extracellular steps convert the procollagen molecule to collagen and incorporate it into stable, cross-linked collagen fibrils. The pro α-chains are initially synthesized with additional signal peptide at the aminoterminal end. The signal peptide directs movement of the polypeptides into the rough endoplasmic reticulum and is then cleaved off.

Many enzymes and several chaperones such as BiP and HSP47 are involved in the posttranslational modification, folding and processing of the procollagen molecules. The main modification steps after the collagen is synthesized in the endoplasmic reticulum are:

1. Certain prolyl and lysyl residues are hydroxylated. About half of the proline residues in the Y position of the Gly-X-Y triplets are converted to 4-hydroxyproline by the enzyme prolyl-4-hydroxylase (MW 240 000, tetramer). Cofactors of hydroxylation are Fe^{++}, α-ketoglutarate, oxygen and ascorbic acid. The extent of hydroxylation depends on the species, and the cell and tissue type, and may change during development and ageing or under pathological conditions. A few proline residues (all in the X position) may also be converted to 3-hydroxyproline by the enxyme prolyl-3-hydroxylase. Accordingly, a proportion of lysine residues is hydroxylated by lysyl hydroxylases (Von der Mark 1999).
2. Some hydroxylysyl residues are glycosylated to galactosylhydroxylysine and glucosylgalactosylhydroxylysine. Two specific enzymes, galactosyltransferase and a glucosyltransferase catalyze these glycosylations. The first of these enzymes adds galactose to the hydroxylysyl residues and the second adds glucose to the galactosylhydroxylysyl residues.
3. The aminoterminal propeptide is phosphorylated to serine residues. The carboxyterminal propeptide is glycosylated to asparagine residues with mannose, glucosamine and galactosamine.
4. Disulphide bonds are formed and chains associated. The C-propeptides fold into the correct conformation stabilized by intrachain disulphide bonds and assemble three α-chains to the procollagen trimer. The formation of a native disulphide bond requires the catalysis by the enzyme disulphide isomerase (PDI), which is identical to the β-

subunit of prolyl-4-hydroxylase. Only procollagen chains containing eight cysteines in the C-propeptide are able to form homotrimers. Procollagen chains lacking two or three of the eight cysteine residues such as proα2(I) can form only heterotrimers.

5. Triple helix is formed. Rate limiting for the folding of the triple helix is the cis-trans-isomerization of prolyl peptide bonds in the α-chains, which is catalyzed by the enxyme peptidyl-prolyl cis-trans-isomerase (PPI). After alignment of the C-propeptides, triple helix formation starts from the C-terminus and progresses toward the N-terminus.
6. Procollagen is secreted into the extracellular matrix.
7. Procollagen is converted to collagen. Following secretion of procollagens in secretory vesicles, the C- and N-propeptides of type I collagen are cleaved off by specific proteases. Both collagen N-proteinase and procollagen-C-proteinases belong to a family of Zn2+-dependent metalloproteinase M12. Also other proteinases seem to be able to cleave procollagen propeptides, e.g. cathepsin D (Von der Mark 1999).
8. Collagen molecules aggregate and the cross-links are formed. Lysyl oxidase catalyzes the cross-link formation.
9. Some aspartyl residues in the telopeptides are isomerized (α→β) and racemized (L→D).

2.5 Cross-linking of type I collagen

The strength of the collagen fibres depends on the formation of covalent cross-links between the telopeptide and adjacent helical domains of collagen molecules. Type I collagen has four cross-linking sites: one in each telopeptide and two others at the sites in the triple-helical domain at residues 87 and 930. An inborn defect with inhibited collagen cross-linking leads to severe diseases, e.g. Ehlers-Danlos syndrome, Marfan syndrome, Menke's disease or X-linked form of cutis laxa.

2.5.1 A short history of cross-link research

Verzar demonstrated about 40 years ago (1964) that collagen fibrils are bound together by cross-links, although the nature of the cross-links was unknown. He proposed that collagen contains two types of cross-links: enzymatically formed and indirectly and non-specifically formed. This indirect form is now known as glycation.

The only enzyme needed for forming the cross-links is lysyl oxidase, which was found by Siegel in 1976.

Before that the reducible cross-links had already been found (Bailey & Lister 1968). They could be isolated and identified only after chemically reducing them in the protein with radioactive sodium borohydride $NaBH_4$. The researchers wondered how the reducible cross-links disappeared over time. Robins *et al.* (1973) confirmed this reduction. The answer was found when the trivalent cross-links were detected. A

fluorescent cross-linking amino acid was isolated from ox tendon and bone collagen and shown to be a 3-hydroxypyridinium derivative that had three amino acid side chains (Fujimoto *et al*. 1977, Fujimoto *et al*. 1978). Housley and Tanzer (1975) were the first to detect histidine to their great surprise in cross-link structure isolated from bovine skin collagen. The collagen digests were also found to give pink colour with Ehrlich's reagent (p-dimethylaminobenzaldehyde) in acid solution, which led to the assumption that pyrrole could be a cross-link in collagen (Scott *et al*. 1981, 1983). There are still open questions and unknown structures in this field.

2.5.2 The divalent cross-links

The distribution of hydrophobic and charged amino acids in the type I collagen α chains determines that the collagen molecules crawl at the right place with respect to each other. Then collagen molecules aggregate so that each molecule is longitudinally displaced about one quarter of its length relative to its nearest neighbour, a requirement for cross-link formation (Prockop *et al*. 1979).

The cross-linking is based upon aldehyde formation from the single telopeptide lysine or hydroxylysine residues, which the lysyl oxidase deaminates. Lysyl oxidase binds to a highly conserved sequence (Hyl-Gly-His-Arg) opposite the N- and C-terminals of an adjacent quarter-staggered molecule. The cross-linking pathway is segregated into two classes depending on the hydroxylation of lysine residue: the allysine (the lysine derived aldehyde) route which predominates in skin, cornea and sclera, and the hydroxyallysine (the hydroxylysine derived aldehyde) route which predominates in bone, cartilage, ligaments and tendons (Eyre *et al*. 1984, Eyre 1987). The basis for this is the different lysyl hydroxylase activity, which seems to determine the cross-link type (e.g. Henkel *et al*. 1987). Different isoforms of lysyl hydroxylase in different tissues are proposed (Valtavaara *et al*. 1997), since the first purified lysyl hydroxylase failed to hydroxylate telopeptide regions (Royce & Barnes 1985).

First, divalent cross-links are formed between the collagen telopeptide region and the helical region. The allysine and the hydroxyallysine pathways lead to different divalent and trivalent cross-links as shown in Figure 1. Most of the divalent cross-links can be reduced with borohydride and are called reducible cross-links (Robins & Bailey 1975). When two aldehydes condense, the aldol condensation product (ACP) is formed.

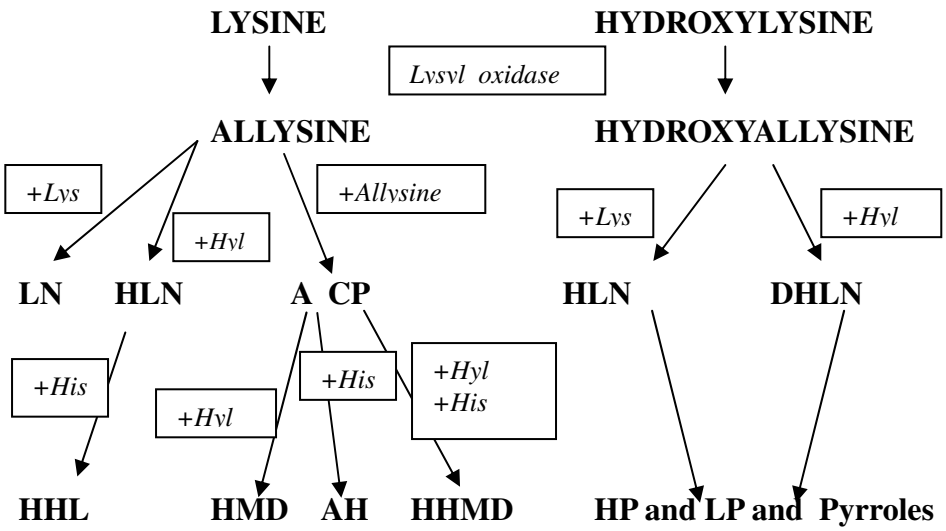


Fig. 1. Allysine and hydroxyallysine cross-links (modified from Eyre *et al.* 1984): LN lysinonorleucine, HLN hydroxylysinonorleucine, ACP aldol condensation product, HHL histidinohydroxylysinonorleucine, HMD hydroxymerodesmosine, AH aldol histidine, HHMD histidinohydroxymerodesmosine, DHLN dihydroxylysinonorleucine, HP hydroxylysyl pyridinoline, LP lysyl pyridinoline.

2.5.3 The mature, trivalent cross-links

The mature cross-linking residues on the hydroxylysine aldehyde pathway are trivalent 3-hydroxypyridinium residues, lysyl pyridinoline (LP or Dpd or D-Pyr) and hydroxylysylpyridinoline (HP or Pyr). Both compounds are fluorescent (Fujimoto *et al*. 1977) with characteristic excitation and emission spectra (325 nm/405 nm in neutral pH). Their second property is UV photolysis of both compounds (Eyre 1987). HP and LP are both widely distributed in different collagen types all over the body, but LP is, however, relatively more abundant in bones and dentin. Also the presence of pyrrole has been confirmed especially at N-terminal telopeptide of type I collagen (Kuypers *et al*. 1992, Hanson & Eyre 1996). These trivalent cross-links connect together two telopeptide chains and one helical domain in a separate collagen molecule (Kuboki *et al*. 1981). In bone, a remarkable portion of cross-links remains as borohydride-reducible divalent cross-links or even non-cross-linked (Eyre 1987). The pyrrole cross-links (Scott *et al*. 1983) are labile in their nature e.g. towards acid hydrolysis and atmospheric oxidation.

The fibrils of type I collagen in skin have a specific cross-link called histidinohydroxylynonorleucine (HHL) in the carboxyterminal telopeptide region (Yamauchi *et al*. 1987). During normal ageing the content of HHL-cross-linked type I collagen increases in skin (Yamauchi *et al*. 1988). In addition to HHL, an another

trivalent cross-link, pyridinoline analogue (PA) or 3-deoxypyridinoline, has been suggested to exist in skin in the same molecular location (Barber *et al*. 1982, Tilson *et al*. 1985).

When the immunoassays for telopeptides have been applied for purifying cross-linked peptides, and these have then been analyzed for cross-link content it has been found that some unknown cross-links must exist (Fledelius *et al*. 1997).

2.6 Degradation of type I collagen

2.6.1 Principles of the degrading enzymes

Type I collagen together with other extracellular matrix molecules is degraded in normal remodelling associated with physiological processes such as morphogenesis, growth, wound healing and physiological bone turnover during calcium release. In addition, extracellular matrix is degraded during pathological processes, such as arthritis, osteolysis or spreading of tumour cells. Also cell-cell and cell-matrix interactions include extracellular matrix proteolysis.

The four major enzyme classes are the aspartate, cysteine, serine and matrix metalloproteinases. Serine and cysteine proteases utilize their HO- and HS-side chains, aspartate proteases aspartate residues and metalloproteases heavy metals, to immobilize and polarize a water molecule so that the oxygen atom in water becomes a nucleophile, which attacks the carbonyl-carbon of an amide bond to be cleaved in the type I collagen molecule.

2.6.2 An aspartic proteinase, Cathepsin D

This aspartic proteinase has optimal proteolytic activity at acid pH between 3 and 5 and it shows only little activity at neutral pH. It degrades gelatin and collagen telopeptides, but can not cleave native triple helical collagen. It is involved both in the extracellular degradation and intracellular breakdown of phagocytosed matrix molecules (Clark & Murphy 1999). Cathepsin D is an important proteolytic enzyme in breast cancer cells, where it is contained in large mobile intracellular acidic vesicles (Montcourrier *et al*. 1990). Cathepsin D might also be involved in the release of carboxyterminal propeptide of type I procollagen (Helseth & Veis 1984).

2.6.3 Cysteine proteases

2.6.3.1 Cathepsins B, L and S

Cathepsins B, L and S are lysosomal enzymes with acidic pH optima, and degrade intracellularly phagocytosed matrix molecules. They can also act as extracellular proteinases at near to neutral pH. The cathepsins B, L and S can cleave gelatin and the telopeptides of type I and II collagens and depolymerize them. The cathepsins are inhibited by protease inhibitors called cystatins.

Cathepsin B has two forms, the lysosomal active form, which is unstable at neutral pH, and a higher molecular weight form, which remains stable at neutral pH and is secreted extracellularly from stimulated connective tissue cells and activated macrophages. Cathepsin B is found in osteoblasts and it is active in rheumatoid arthritis.

2.6.3.2 Cathepsin K

The major collagen degrading enzyme in osteoclasts is a cysteine protease called cathepsin K (Delaissé *et al*. 2000). It has long been assumed that osteoclasts must produce an acid collagenase able to degrade the organic matrix in the acidic microenvironment (pH 3.5–4) of the resorbing lacunae (Blair *et al*. 1986). At 1993 Blair *et al*. isolated an acid proteinase from avian osteoclasts with properties corresponding those of cathepsin K, a finding that has not been referred to in the following cathepsin K papers. Before that some kind of cathepsin L-like activity had been detected in osteoclasts by Delaisse *et al*. (1991). Later the cDNA clones for this cysteine protease were cloned independently by several groups, first in the rabbit (Tezuka *et al*. 1992, 1994) and later from human osteoclast cDNA library (Brömme & Okamoto 1995, Drake *et al*. 1994, Inaoka *et al*. 1995, Li *et al*. 1995, Shi *et al*. 1995). The novel protease was first named either cathepsin O, K, O2, X or OC2 by different groups. The Nomenclature Committee of the International Union of Biochemistry and Molecular Biology finally assigned the name cathepsin K (EC 3.4.22.38) to this novel protease. Cathepsin K is initially transcribed and translated as an inactive precursor, preprocathepsin K (MW 37 000), typical also for the other members of the cysteine protease family.

Cathepsin K belongs to a papain superfamily of cysteine proteinases. The other families of the cysteine proteinase group are bleomycin hydrolase and calpain groups. The phylogeny of the papain group indicates that many families diverged almost simultaneously early during eukaryotic evolution by gene duplications and mutations affecting the residue charges of the enzyme (Berti PJ & Storer AC 1995, Hughes AL 1994). The greatest homology (56%) occurs between cathepsin K and S (Bossard *et al*. 1996).

Cathepsin K is highly expressed in osteoclasts and osteoclastomas (Brömme & Okamoto 1995, Inaoka *et al*. 1995). Unlike other lysosomal cysteine proteinases, which show wide tissue distribution and expression, cathepsin K is highly cell and tissue

specific, concentrating in the osteoclasts of the resorbing bone, also during foetal development (Dodds *et al*. 1998). Other tissues, such as spleen, liver, kidney, lung, brain, heart, alveolar macrophages, bone stromal cells and inflammatory synovial cells show practically none or only slight cathepsin K expression. This has been confirmed several times by many groups by immunolocalization (Drake *et al*. 1996), *in situ* hybridization (Rantakokko *et al*. 1996) and fluorescence microscopic studies (Kamiya *et al*. 1998). Cathepsin K is expressed specifically at the cell surface adjacent to bone (Littlewood-Evans 1997, Kamiya *et al*. 1998), and in the osteoclasts associated with bone surfaces (Votta *et al*. 1997). Cathepsin K is concentrated as band-like deposits along the ruffled border-like structure of the osteoclasts facing the bone resorption lacuna, and in vesicles, granules and vacuoles close to the ruffled border and perinuclear regions of resorbing osteoclast (Yamaza *et al*. 1998). Chondroclasts facing the resorption lacunae of the cartilage and mononuclear preosteoclasts show cathepsin K immunoreactivity as well (Yamaza *et al*. 1998). The other bone cells, such as osteoblasts, osteocytes and bone marrow cells, show no cathepsin K expression (Littlewood-Evans *et al*. 1997 A, Yamaza *et al*. 1998). Primary tumour cells as well as invading cells in bone metastases of breast cancer also express cathepsin K (Littlewood-Evans *et al*. 1997 B). Normal arteries contain little or no cathepsin K, but macrophages and intimal smooth muscle cells in atheroma contain abundant immunoreactive cathepsin K (Sukhova *et al*. 1998).

Though cathepsin K is a highly bone-specific enzyme, the different bones contain variable amounts of cathepsin K activity. The data originating from the cathepsin K knock-out mice demonstrate that the parts of skeleton that are involved in rapid bone remodelling (for example, the long bones and vertebrae) contain more cathepsin K than the bones known to have a low resorption rate, such as calvariae (Gowen *et al*. 1999, Everts *et al*. 1999). On the other hand, cathepsins S, B and L, which have also been proposed to be involved in bone resorption and remodelling, are either expressed at very low levels or not at all in osteoclasts (Drake *et al*. 1996).

Cathepsin K is autoactivated in acidic pH and elevated temperature (Bossard *et al*. 1996) by breaking some salt bridges of the molecule (LaLonde *et al*. 1999). The maximal enzyme activity is achieved at assay conditions near pH 5.5 (Bossard *et al*. 1996), while the pH around the ruffled border is known to be lower. Cathepsin K has unique property among the collagenolytic enzymes to cleave collagen both in the helical (Garnero *et al*. 1998) and telopeptide (Brömme *et al*. 1996) regions. Some cleavage sites within type I and type II collagen helical and telopeptide regions have been determined (Garnero *et al*. 1998, Kafienah *et al*. 1998, Atley *et al*. 2000). Studies with synthetic substrates of general structure Cbz-P_3-P_2-P_1-AMC show that an amino acid with hydrophilic side chain such as Arg or Lys in P_1 along with an amino acid having a small, hydrophobic side chain within P_2 are greatly favoured as substrates of cathepsin K (Bossard *et al*. 1996). One of the most favoured substrates for cathepsin K seems to be Cbz-Leu-Arg-AMC (Bossard *et al*. 1996). Substrates with proline at P_2 position have been identified as specific for cathepsin K (Aibe *et al*. 1996), and also leucine, phenylalanine or valine are accepted (Brömme *et al*. 1996). Glysine is accepted in the P_1 site, and this may explain why cathepsin K is able to cleave also the triple-helical part of collagen (Atley *et al*. 2000).

The importance of cathepsin K for bone resorption and the individual have been demonstrated by 1) cathepsin K inhibitor studies (Inui *et al*. 1997), 2) pycnodysostosis, a rare human disease due to cathepsin K deficiency (Gelb *et al*. 1996, Hou *et al*. 1999) and

3) cathepsin K knock-out mice (Saftig *et al*. 1998, Gowen *et al*. 1999). Before cathepsin K was discovered, some cysteine proteinase inhibitors, such as leupeptin, E-64 and cystatin, were shown to inhibit bone resorption (Delaisse *et al*. 1984, Lerner *et al*. 1992, Delaisse *et al*. 1987). Subsequently, Votta *et al*. (1997) have found that peptide aldehyde inhibitors, especially Cbz-Leu-Leu-Leu-H, inhibit bone resorption both *in vitro* and *in vivo*. Pycnodysostosis is characterized by dwarfism, wide cranial sutures, acro-osteolysis of distal phalanges, dental anomalies, increased bone density (osteopetrosis) and fragility and characteristic facial appearance resulting from calvarial bossing, loss of mandibular angle and dental abnormalities due to a mutation in the cathepsin K gene. Since the initial description in 1962, there have been about 100 cases listed worldwide (Ho *et al*. 1999). Missense, nonsense or stop codon mutations in the cathepsin K gene have been found in pycnodysostosis patients (Johnson *et al*. 1996, Gelb *et al*. 1996, Ho *et al*. 1999). A mutation in the cathepsin K gene may lead either to a reduced cathepsin K level or a total absence of cathepsin K. Even a significantly reduced cathepsin K level does not lead to any phenotypic effects, while the total absence of the cathepsin K leads to pycnodysostosis (Ho *et al*. 1999). Cathepsin K knockout mice have osteopetrosis in long bones, especially in distal femur, lumbar vertebrae and increased trabecular and cortical bone mass compared with the wild type (Gowen *et al*. 1999). They also have accumulation of thickened calcified cartilage septa in the growth plates, their osteoclasts do not resorb demineralized bone efficiently and they develop splenomegaly due to suppressed extramedullary haematopoiesis and reduced bone marrow cellularity. However, a loss of function of cathepsin K does not universally affect the ability of osteoclasts to resorb bone, neither does it affect all bones equally. In summary, the lack of cathepsin K leads to a substantially decreased rate of bone resorption but not a complete cessation of the process, indicating that some other enzymes are also involved in the process (Nishi *et al*. 1999).

Because of the essential role of cathepsin K for bone resorption, inhibitors for this enzyme are under investigation as therapeutic agents (Thompson *et al*. 1997, Votta *et al*. 1997). The most effective inhibitors seem to be tetrahedral intermediates that resemble those that occur during substrate hydrolysis. The inhibitors are able to bind and span the catalytic site of the enzyme either reversibly or irreversibly (Thompson *et al*. 1997).

2.6.3.3 Serine proteinases

The polymorphonuclear leukocytes of blood (PML) contain neutrophil elastase, cathepsin G and proteinase 3. The contents of their granules may be discharged into the extracellular space during inflammation. These three enzymes have a pH optimum at pH 7 to 9, and they can degrade elastin, telopeptides of fibrillar collagens and type IV collagen, matrix glycoproteins, gelatin and proteoglycan core protein. These enzymes are inhibited by α2-macroglobulin and α1-proteinase inhibitor, α1-antichymotrypsin, leukocyte proteinase inhibitor and thrombospondin 1 (Clark & Murphy 1999, review).

Also serine proteases plasmin, tissue plasminogen activator and urokinase-type plasminogen activator, plasma kallikrein, tissue kallikrein, tryptase and chymase are involved in ECM degradation by either straight degrading of the ECM components or by activating the various members of matrix metalloproteinases (Clark & Murphy 1999, Chapman *et al*. 1997).

2.6.4 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are members of a subfamily of proteinases, which includes collagenases (MMP-1, -8, -13 and –18), stromelysins (MMP-3, -10, -11, -7 and –12), gelatinases (MMP-2 and –9) and membrane type MMPs (MT-MMPs) (MMP-14, -15, -16, -17) (Table 1). Some of the newly described MMPs (MMP-4, -5, -6, -19 and -20) can not be included in any of these groups. Together they are able to degrade most of the proteins in the extracellular matrix. As seen in Table 1, almost all of them are able to degrade some types of collagen and gelatin, and most of them, especially MT-MMPs, activate other MMPs.

The existence of collagen degrading collagenases has been known for three decades (Harris & Krane 1974). To find an enzyme there are nowadays two possibilities: cDNA cloning and conventional biochemical purification. Most of the MMPs have been cloned, and if they were first purified, they have usually been cloned later. The first of them to be cloned was MMP-1 (Goldberg *et al*. 1986), thought the presence of this enzyme had been known much earlier.

The genes for MMPs code 5 different domains. At least three of these domains are included in every MMP, even in the simplest one, MMP-7 or matrilysin. The domains are from the N-terminal to the C-terminal end: a signal peptide, a propeptide, a catalytic domain with the highly conserved zinc-binding site, a hinge region and a haemopexin like domain (Kähäri & Saarialho-Kere 1997). In addition, MT-MMPs contain a transmembrane domain in the haemopexin-like domain in the C-terminal end and the gelatinases fibronectin type II like inserts within the catalytic domain (Collier *et al*. 1991). The collagenase and gelatinase subfamilies are supposed to be evolved by duplication on separate chromosomes, with the ancestor of the two type IV collagenases acquiring the fibronectin-like domain prior to duplication (Collier *et al*. 1991).

The knowledge of locations of MMPs in the body is collected in Table 1.

Table 1. The main cells producing different MMPs in the body.

Enzyme	MMP	Cells
Collagenases		
Interstitial collagenase, Collagenase-1	MMP-1	Keratinocytes, endothelial cells, monocytes, macrophages, neutrophils, chondrocytes, osteoblasts, tumor cells
Collagenase-2, Neutrophil collagenase	MMP-8	Neutrophils, articular cartilage chondrocytes (1) Rheumatoid synovial fluid cells and human endothelial cells (2)
Collagenase-3	MMP-13	Breast cancer cells (3) Squamous cell carcinoma cells (4) Fibroblast-and macrophage-like mononuclear cells in the synovial lining and stroma, especially in rheumatoid arthritis (5) Osteoarthritic cartilage cells (6, 7) Foetal chondrocytes, foetal osteoblasts and periosteal cells (8) Transformed epidermal keratinocytes (9)
Stromelysins		
Stromelysin-1	MMP-3	Stimulated rabbit synovial fibroblasts (10), cartilage (11)
Stromelysin-2	MMP-10	Cloned from rat fibroblast cDNA library (12)
Stromelysin-3	MMP-11	Fibroblasts of involuting mammary gland, placenta and uterus cells, cells of developing limb bud (13)
Matrilysin	MMP-7	Bone-marrow derived promonocytes, blood monocytes, monocyte-derived macrophages (14) Epidermal cells of developing skin and in tumour cells of cutaneous malignancies (15) Exocrine epithelial cells throughout the body (16) In variety of carcinomas ranging from adenomas to carcinomas and adenocarcinomas of the breast, colon, prostate, stomach, upper aerodigestive tract, lung, skin (17)
Matrilysin -2	MMP-26	Placenta, uterus, malignant tumours (18,19)
Metalloelastase	MMP-12	(20-22)
Gelatinases		
Gelatinase A	MMP-2	Fibroblasts in tumour invasion and metastasis
Gelatinase B	MMP-9	Osteoclasts, osteoclastomas (23-25) Osteoclasts of Paget's disease Osteoclast of developing bone (26) Neutrophils, monocytes

References 1. Cole et al. 1996, 2. Hanemaaijer et al. 1997, 3. Freije et al. 1994, 4. Johanson et al. 1997a, 5. Lindy et al. 1997, 6. Mitchell et al. 1996, 7. Wernicke et al. 1996, 8. Johanson et al. 1997b, 9. Johansson 1997c, 10. Chin et al. 1985, 11. Wilhelm et al. 1993, 12. Breatnach et al. 1987, 13. Lefebvre et al. 1992, 14. Busiek et al. 1992, 15. Karelina et al. 1994, 16. Saarialho-Kere et al. 1995, 17. Wilson & Matrisian 1996, 18.Uria A & Lopez-Otin C 2000, 19. Park et al. 2000, 20. Banda & Werb 1981, 21. Shapiro et al. 1992, 22. Shapiro et al. 1993, 23. Wucherpfenning et al. 1994, 24. Okada et al. 1995, 25. Tezuka et al. 1994, 26. Reponen et al. 1994, 27. Nomura et al. 1995, 28. Okada et al. 1995, 29. Sato et al. 1997, 30. Will & Hinzmann 1995, 31. Takino et al. 1995, 32. Puente et al. 1996, 33. Llano et al. 1999 34. Velasco et al. 2000 34. Velasco et al. 2000, 35. Pendas et al. 1997, 36. Llano et al. 1997, 37. Bartlett et al. 1996, 38. Velasco et al. 1999, 39. Lohi et al. 2001.

Enzyme	MMP	Cells
Membrane-type MMPs		
MT1-MMP	MMP-14	Carcinoma cells in MMP-2 positive cases (27) Tumour fibroblasts (28) Osteoclasts (29)
MT2-MMP	MMP-15	Human lung cDNA library (30)
MT3-MMP	MMP-16	Human placenta cDNA library (31)
MT4-MMP	MMP-17	Breast carcinoma cDNA library (32)
MT5-MMP	MMP-24	Brain, brain tumours (astrocytomas, glioblastomas), kidney, pancreas, lung (33)
MT6-MMP	MMP-25	Leucocytes, lung, spleen, colon carcinoma, brain tumours (34)
Other MMPs		
Unnamed	MMP-19	Liver cDNA library (35)
Enamelysin	MMP-20	Odontoblastic cells (36) Porcine enamel organ (37)
	MMP-23	Reproductive tissues (ovary, testis, prostate) (38)
Epilysin	MMP-28	Testis, keratinocytes, in response to injury (39)

References 1. Cole et al. 1996, 2. Hanemaaijer et al. 1997, 3. Freije et al. 1994, 4. Johanson et al. 1997a, 5. Lindy et al. 1997, 6. Mitchell et al. 1996, 7. Wernicke et al. 1996, 8. Johanson et al. 1997b, 9. Johansson 1997c, 10. Chin et al. 1985, 11. Wilhelm et al. 1993, 12. Breatnach et al. 1987, 13. Lefebvre et al. 1992, 14. Busiek et al. 1992, 15. Karelina et al. 1994, 16. Saarialho-Kere et al. 1995, 17. Wilson & Matrisian 1996, 18.Uria A & Lopez-Otin C 2000, 19. Park et al. 2000, 20. Banda & Werb 1981, 21. Shapiro et al. 1992, 22. Shapiro et al. 1993, 23. Wucherpfenning et al. 1994, 24. Okada et al. 1995, 25. Tezuka et al. 1994, 26. Reponen et al. 1994, 27. Nomura et al. 1995, 28. Okada et al. 1995, 29. Sato et al. 1997, 30. Will & Hinzmann 1995, 31. Takino et al. 1995, 32. Puente et al. 1996, 33. Llano et al. 1999 34. Velasco et al. 2000 34. Velasco et al. 2000, 35. Pendas et al. 1997, 36. Llano et al. 1997, 37. Bartlett et al. 1996, 38. Velasco et al. 1999, 39. Lohi et al. 2001.

The MMPs are regulated at transcriptional and post-transcriptional levels. First, their genes are selectively expressed in specific cell types (Table 1) where they are induced by growth factors, cytokines, oncogenes (c-fos, c-jun, c-ets), tumour promoters, hormones, chemical agents (phorbol esters, actin stress fiber disrupting drugs etc.) or physical stress. For example, ultraviolet B irradiation up-regulates MMP-1, MMP-3 and MMP-9 expression in human dermis. Also cell-matrix and cell-cell interactions induce MMP expression by e.g. integrins as mediators. Some MMPs are co-ordinately regulated (Matrisian 1992, Curran & Murray 1999 and Nagase & Woessner 1999).

Most MMPs are secreted from the cells as inactive zymogens, containing the propeptide domain. The secreted pro-MMPs can be activated *in vitro* by proteinases and inorganic compounds such as SH-reactive agents, mercurial compounds, reactive oxygen, and denaturants. During activation the interaction between a zinc atom and a cysteine residue in the conserved PRCGVPD region is disrupted and after this the propeptide is cleaved off in a stepwise manner. *In vivo* the MMPs are activated by tissue or plasma proteinases, opportunistic bacterial proteinases or by the uPA/plasmin system (Hideaki & Woessner 1999, Matrisian 1992, Curran & Murray 1999). The proteolytic events between the enzymes to be activated are shown in Figure 2.

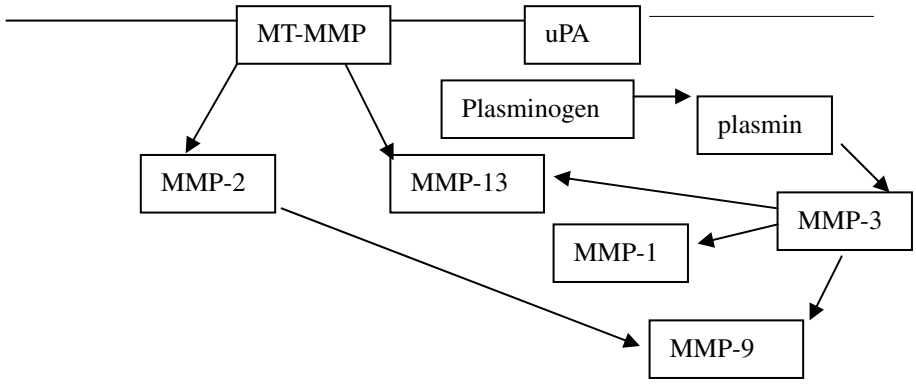


Fig. 2. The activation cascade of the MMPs as modified from Curran & Murray 1999.

The third part of MMP regulation includes their inhibition by non-specific inhibitors such as α2-macroglobulin and by specific tissue inhibitors of metalloproteinases (TIMPs). TIMP-1 is a 28 000 glycoprotein and TIMP-2 a 20 000 unglycosylated protein. There is 40 % sequence identity between TIMP-1 and TIMP-2. TIMP-3 and TIMP-4 have also been described. TIMP-2 is often in complex with MMP-2 and does not prevent gelatinolysis and a second molecule is needed to inhibit proteolysis. Maybe TIMP-2 binds to a site distinct from the active site stabilizing the enzyme. Synthetic MMP inhibitors are targets of intensive research as potential drugs in many conditions (Hidalgo & Eckhardt 2001).

TIMPs are expressed co-ordinately with MMPs. TIMPs are able to inhibit the active enzyme, but not to inhibit autoactivation. Thus the enzyme degrades the substrate only a limited amount before being inactivated by TIMPs. Thus the ECM degradation remains tightly controlled (Matrisian 1992).

2.7 Assessing the rate of type I collagen synthesis

The procollagen propeptides, which guide assembly of the triple helix, are cleaved from the newly formed molecule (Figure 3) in a stoichiometric relationship with collagen biosynthesis.

The carboxyterminal propeptide of type I procollagen (PICP) with a molecular mass of 100 000, is a trimeric globular glycoprotein. Disulphide bonds stabilize it. Its half-live in serum is 6–8 min and it is cleared in the liver endothelial cells by the mannose receptor. There have been altogether 4 immunoassays for PICP (e.g. Melkko *et al*. 1990).

The procollagen type I aminoterminal propeptide (PINP), a 35 000 globular protein, contains an internal region of 17 Gly-X-Y- triplets. It is cleared from circulation by the scavenger receptors of liver endothelial cells. The assay for PINP (Melkko *et al*. 1996) has been shown to be a very valuable assay for collagen synthesis in various conditions.

Despite the fact that PICP and PINP are derived from the same molecule, no constant temporal or disease-specific correlation exists between these molecules. E.g. in stable breast cancer their correlation is linear, but in aggressive breast cancer the PICP:PINP ratio is low (Jukkola *et al*. 1997).

2.8 Biochemical alternatives to measure collagen degradation

When scientists and physicians want to measure collagen metabolism, they are usually interested in metabolic bone diseases and osteoporosis. However, the quantification of the markers mirroring collagen metabolism is applicable in various other conditions as well. The major concern is: which one of the markers gives the most reliable information in each particular condition, e.g. in metabolic bone disease or in tumour metastasis?

In spite of several disadvantages the oldest and most applied method to measure collagen degradation (e.g. bone turnover) is to quantitate urine hydroxyproline. It correlates with calcium aggregation and bone resorption in many clinical conditions, e.g. in Paget's disease of bone and osteoporosis and other changes in bone turnover (Calvo *et al*. 1996). Nevertheless, althought providing useful information, it still is far from being the ideal method, since only 10 % is excreted in the urine. Further, a proportion of hydroxyproline derives from collagen synthesis, since PINP contains a collageneous domain. In addition, the C1q component of complement contains hydroxyproline in its structure, which in inflammatory situations such as rheumatoid arthritis may significantly contribute to the urinary excretion of hydroxyproline.

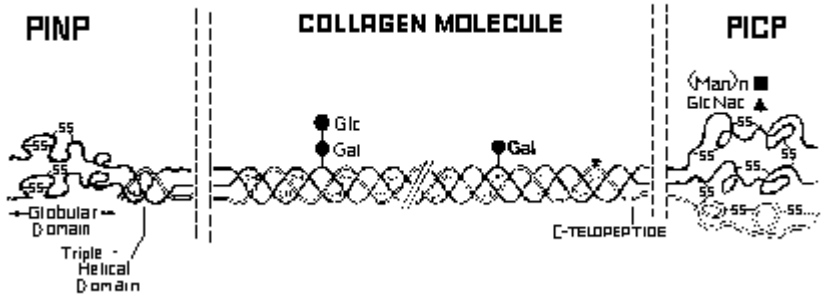


Fig. 3. Structure of type I procollagen (modified from Robey 1995).

The assay of galactosyl hydroxylysine (GHYL) has similar problems as urinary hydroxyproline. The analysis method is tedious and very complicated. For this reason it is not widely used and needs further development and validation.

The next achievements in the field were the cross-link analyses. The main advantage of these assays was that the cross-links derive form the old, not from the newly synthesized collagen. Several assays both for urine and serum Pyr and Dpd analyses have been developed, including free and peptide bound pyridinolines and HPLC- and ELISA methods. The osteoclasts release the peptide-bound form but not the free form (Apone *et*

al. 1997). The cleavage of the peptide-bound to the free form appears to be a rate-limiting, saturable process occurring in the kidney (Collwell & Eastell 1996). Further, the bisphosphonate and estrogen treatments have different effects on their excretion, bisphoshonates reducing only peptide-bound and estrogen treatment both free and peptide-bound cross-links (Garnero *et al*. 1995). In general, Dpd is referred to as deriving mostly from bones (e.g. Eastell *et al*. 1997). The free Dpd is not as sensitive to the changes in bone physiology as total Dpd (Rubinacci *et al*. 1999).

The most immunogenic parts of the type I collagen are the telopeptides and there are several immunoassays for their specific measurement.

The ELISA-assay for the measurement of the N-terminal telopeptide structures in urine (Ntx) is based on monoclonal antibodies raised against pyridinoline fluorescent N-terminal structure purified from the urine of a patient with Paget's disease (Hanson *et al*. 1992). The assay was found to be sensitive for osteoclastic bone resorption. The effect of cathepsin K on the NTx assay has been reported by Atley *et al*. (2000). Cathepsin K releases a neoepitope required by the NTx assay (Hanson & Eyre 1996) when it cleaves a peptide bond GVG/L in the N-terminal telopeptide of α2(I)-chain. In fact, Atley et al (2000) reported that recombinant human cathepsin K is highly active in releasing the NTx epitope with a 100% yield from bone type I collagen. Other cathepsins are also capable of producing the NTx antigen, but not to the same extent as cathepsin K. The matrix metalloproteinases do not release Ntx antigen (Atley *et al*. 2000).

A new assay (Serum CrossLaps™ One Step ELISA) for the determination of serum concentrations of the β-isomerized C-telopeptide of type I collagen has recently been developed (Rosenqvist *et al*. 1998) and applied clinically (Christgau *et al*. 1998). This assay is performed as a monoclonal sandwich assay in a one-step procedure and is based on a monoclonal antibody that exclusively recognizes the isomerized β-aspartate (D) form of the EKAHβDGGR epitope of the α1-chain of human type I collagen (Fledelius *et al*. 1997). This sequence contains the lysine residue that participates in the intermolecular cross-linkage of the collagen chains. Cathepsin K is also able to generate fragments that are recognized by the CrossLaps ELISA (Garnero *et al*. 1998). Because the CrossLaps assay requires free C-terminal Arg in the EKAHDGGR-peptide fragment of type I collagen (Bonde *et al*. 1997), the authors have deducted that there must be a cleavage between Arg^{22} and Tyr^{23} in the cathepsin K released peptide (Garnero *et al*. 1998).

CrossLaps ELISA reacts sensitively to changes both in physiological bone turnover and due to therapies, e.g. during hormone replacement therapy and bisphosphonate treatment (e.g. Bjarnason & Christiansen 2000, Christgau 2000). The circadian variation within an individual is remarkable in CrossLaps, having a peak at night (Wichers *et al*. 1999), and it is augmented in non-fasting individuals.

The assay for human carboxy-terminal telopeptide (ICTP) was developed in 1993 (Risteli *et al*. 1993). The assay uses polyclonal antibodies and a tracer purified from human bones. The ICTP assay is introduced elsewhere in this thesis.

3 The aims of the study

This study was designed to find answers to the following questions:

1. Why is the assay for the carboxyterminal telopeptide of type I collagen, ICTP, insensitive to physiological changes in bone turnover, although it has been shown to be a reliable marker in several pathological conditions
   - by determining the antigenic determinant of the ICTP assay,
   - by studying how the most prominent collagen degrading enzyme in osteoclasts, cathepsin K, affects the immunoreaction of the ICTP antigen,
   - by studying how the matrix metalloproteinases affect the immunoreaction
   - by studying how the ICTP-assay reacts with trivalently and divalently cross-linked telopeptides.
2. Why is the CrossLaps assay sensitive for physiological bone turnover though both the ICTP and CrossLaps tracer antigens are derived from the same telopeptide region?
3. What kind of carboxyterminal telopeptides can be found in normal and irradiated skin and how they react in the ICTP assay?

And also

4. to develop an assay for bovine ICTP corresponding to that of human.

4 Materials and methods

4.1 Isolation of type I collagen from human and bovine bone and from human uterine leiomyoma and human skin

The insoluble organic matrix of human bone was prepared by demineralization of femoral heads removed during elective hip surgery, as described previously (Risteli *et al*. 1993). The cross-linked type I collagen from human skin was purified from the skin of a patient obtained in breast reduction surgery (Department of Surgery) as described in paper III and from myoma (Department of Obstetrics and Gynegology, Oulu University Hospital). Bovine bone collagen was isolated from a single steer femur obtained from a local abattoir. The tissues were kept at -20°C until used.

When purifying collagen from human or bovine bone, the bones were frozen in liquid nitrogen, cut into small pieces, and pulverized under liquid nitrogen in a mineral mill (Retsch AG, Haan, Germany). The bone powder was defatted in acetone, washed with ethanol to remove it, and air-dried. The non-mineralized soft connective tissue was extracted with 4M guanidine-HCl in 50 mM Tris-HCl buffer, pH 7.4, containing protease inhibitors [aminocaproic acid (0.1 M), benzamidine-HCl (5 mM) and phenylmethylsulfonyl fluoride (1 mM)] for 24 h at 4°C. After centrifugation (15 000 x g for 20 min at 4°C), the insoluble residue was washed several times with distilled water and demineralized three times (all for 24 h at 4°C) with 0.5 M disodium-EDTA (adjusted to pH 7.4 with ammonia), 2 M urea and the protease inhibitors. Between demineralizations, the preparation was centrifuged at 15 000 x g for 20 min at 4°C, and the supernatants were discarded. Finally, the insoluble residue was washed several times with distilled water, dialyzed against distilled water, and lyophilized.

The skin and myoma tissues were cut into small pieces and homogenized with Ultra-Turrax instrument in PBS-Tween 20. The mixture was centrifuged for 20 min, 8000 rpm (Sorvall RC2-B). The insoluble residue from skin and some bone preparations was reduced with $NaBH_4$ (25 mg/ml in 1mM NaOH) for 2 hours and centrifuged for 20 min, 8000 rpm. In one experiment the insoluble residue was reduced with tritiated $NaBH_4$. The residue was washed with water, mixture of 50% acetone and 50% methanol, and finally with absolute ethanol and then lyophilized.

4.2 Isolation of C-terminal telopeptides from the purified collagens

Ten grams of the lyophilized insoluble collageneous residues were suspended in 500 ml of 0.2 M ammonium bicarbonate, pH 7.8, and denatured by heat treatment at 70°C for 1 h. After cooling, the collageneous residue was digested with 200 mg of trypsin (TPCK-treated; Worthington Biochemicals, Freehold, NJ) for 8 h at 37°C. In some series of experiments, the insoluble residue was similarly digested with 8000 units of bacterial collagenase (CLSPA grade; Worthington Biochemicals). The digests were applied into preconditioned 10 g SEP PAK® C_{18} cartridges (Millipore Waters, Milford, MA) in batches of 1 g. The loaded cartridge was washed with 100 ml of distilled water and followed by 100 ml of 30% methanol. The collagen telopeptides were eluted with 100 ml of 70% methanol, and this fraction was lyophilized. Further purification of the cross-linked telopeptides was achieved by gel filtration on a Sephacryl S-100 HR® (Pharmacia, Uppsala, Sweden) column (130 x 2.7 cm) in 0.2 M ammonium bicarbonate buffer, pH 7.8, at a flow rate of 10 ml/h and collecting 5-ml fractions. The elution of the telopeptides was monitored with specific immunoassays, peptide absorbance at 280 nm, and pyridinoline fluorescence (excitation at 325 nm and emission at 405 nm) and Erlich's reaction for pyrroles. When the bovine ICTP antigen was initially purified, weak cross-reactivity with human ICTP antigen was used for monitoring. The fractions containing ICTP antigenicity were pooled and lyophilized. The ICTP was further purified using pH stable, C_8 reverse-phase HPLC (Vydac, Hesperia, CA). The samples were loaded in 0.4% ammonium acetate buffer, pH 7.4, and the bound proteins were eluted with an acetonitrile (75% in the 0.4% ammonium acetate buffer) gradient (0 to 15% in 5 min and 15 to 45% in 5 to 45 min). Finally, the ICTP antigens were purified by anion-exchange HPLC (Protein Pak DEAE 5 PW; Millipore Waters) using 0.02 M ammonium acetate, pH 7.4, containing 1 % isopropanol as a starting buffer and eluting with increasing proportions (0 to 75% in 45 min) of 0.4 M ammonium acetate, pH 7.4. The HPLC runs were continuously monitored for absorbance at 280 nm and for fluorescence (excitation at 325 nm and emission at 405 nm) and with the respective ICTP radioimmunoassay (bovine ICTP, human ICTP or SP4 assay, see below).

The divalently or HHL-cross-linked and non-cross-linked forms of carboxyterminal telopeptides of the human type I collagen α1-chain were purified from the same trypsin digests as the ICTP antigen, first stabilizing the divalent cross-links by reduction with $NaBH_3$ to protect them during EDTA demineralization. The elution of these peptides was monitored with an assay for the synthetic peptide SP4 assay mimicking the carboxy-terminal telopeptide region of type I collagen: SAGFDFSFLPQPPQEKY.

4.3 Chemical characterization of the purified peptides

The purities and sizes of the human and bovine ICTP antigens were verified by SDS-PAGE using 18% gels containing 0.5 M urea or NuPAGE™ 10% Bis-Tris gels (Novex, San Diego, CA, USA). The origin of the ICTP antigens from the carboxy-terminal cross-linking region of type I collagen was verified by the aminoterminal sequence analysis

using a liquid-phase sequencer (Applied Biosystems, Foster City, CA). The protein concentrations of the standard preparations were determined by quantitative amino acid analysis after acid hydrolysis (Biocrom 20, Pharmacia). The molecular weight of the peptides was determined by MALDI-TOF MS (Biflex™, Bruker-Franzen Analytic).

The pyridinoline cross-links in the peptides were detected by their natural fluorescence (excitation at 325 nm, emission at 404 nm). Pyrrolic cross-links were detected by Ehrlich's reaction, adding 0.5 ml aliquots of sample to 0.5 ml of Ehrlich reagent (5% dimethylaminobenzaldehyde and 0.01 % mercuric chloride in 4 M perchloric acid) and measuring the absorbance at 572 nm against a reagent blank after 10 min at room temperature. Although this colour reaction is not sensitive or even specific for pyrrole cross-links, it is useful in detecting pyrrole cross-links in bone collagen digests, which supposedly do not contain other structures giving a similar reaction.

4.4 Production of antibodies

Polyclonal antibodies were raised in New Zealand White rabbits by intradermally injecting in several places 400 µg of thyroglobulin conjugate in 1 ml of 0.9% NaCl mixed with an equal volume of Freund's complete adjuvant. Two milligrams of trypsin-derived bovine ICTP antigen were conjugated to 10 mg of bovine thyroglobulin with 100 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide in 10 mM Na_2HPO_4 buffer, pH 7.5 and the reaction mixture was incubated on ice for 2 h. The preparation was dialyzed against PBS, pH 7.4. Similar booster injections mixed with Freund's incomplete adjuvant were given at 3-week intervals.

4.5 Labelling of the peptides

Ten micrograms of the collagenase liberated ICTP antigen was labelled with 1 mCi of ^{125}I by the chloramine-T method. The labelled antigen was separated from free iodine using a SEP PAK C_{18} cartridge (Millipore Waters), equilibrated in 20% isopropanol and 80% 0.1 M acetic acid, and eluted with 3 ml of 50% isopropanol and 50% 0.1 M acetic acid. The tracers were diluted to a final activity of 50 000 cpm in 200 µl of PBS containing 1 g/L of BSA and 0.04% Tween 20.

4.6 Immunoassays

Quantitative immunoassay for the cross-linked carboxyterminal telopeptide of human (Risteli *et al*. 1993) type I collagen was used (reagents supplied by Orion Diagnostica, FIN-90460, Oulunsalo, Finland). Briefly, 100 µl samples (tissue extracts, diluted enzyme digests or serum samples) were incubated for 2 h at 37°C with 200 µl of iodinated tracer

and 200 µl of an antiserum diluted in 0.5% normal rabbit serum to bind 50% of the tracer. Then 500 µl of a second antibody-polyethylene glycol (PEG) suspension (20 ml of goat antirabbit immunoglobulin antiserum and 150 g of PEG (MW 6000) in 1 L of phosphate-buffered saline (PBS) containing 0.04% Tween-20 was added and vortex-mixed. After 30 min at room temperature, the bound fraction was separated by centrifugation (2000 x g, 30 min, at 4°C). The supernatant containing the unbound tracer was decanted and the radioactivity in the precipitate counted with a 1470 Wizard™ gamma counter (EG & G Wallac, Turku, Finland). The samples were diluted in PBS-Tween. The intra-assay variation for ICTP is between 2.8–6.2 % and inter-assay variation between 4.1–7.9%. The HHL-cross-linked telopeptide variant was assayed essentially similarly to ICTP by an in-house method using a synthetic peptide, SP4 (SAGFDFSFLPQPPQEKY; produced by Neosystem Laboratories, Strasbourg, France), derived from the carboxyterminal telopeptide region of type I collagen as a tracer and standard antigen. The antiserum (#239) used was produced in a rabbit against the divalently cross-linked carboxy-terminal telopeptide antigen of human type I collagen.

The immunoassay for bovine ICTP was performed similarly to the human ICTP assay. The intra- and interassay coefficients of variation were 5.6% (n = 15) and 10% (n = 7), respectively. Known amounts of the purified bovine ICTP were added to a bovine serum sample containing 12.5 µg/L ICTP. In the immunoassay, the mean recovery was 86%.

The concentration of aminoterminal propeptide of type I procollagen (PINP) (Melkko *et al*. 1996, Orion Diagnostica, Espoo, Finland) was analyzed directly in the soluble skin extracts or serum or synovial fluids using 100 µl samples. The inter- and intra-assay variations for PINP varied between 3.1–10.8 and 4.6–10.3%, respectively. PIIINP was measured in duplicate 200 µl aliquots of serum, respectively, and similar diluted (1:1, 1:10 or 1:100 in assay buffer, depending on the sample and assay) aliquots of synovial fluid. Equilibrium radioimmunoassays for the human antigens were used (Risteli *et al*. 1988) with reagents supplied by Orion Diagnostica. The inter- and intra-assay variations are 5 % and 4 % for PIIINP, respectively.

TIMP-1 and TIMP-2 were measured by enzyme-linked immunosorbent assay (ELISA) using polyclonal antibodies produced in chicken. A peroxidase labelled anti-chicken-IgG was used for detection, and the colour formation was measured at 450 nm. For detection of the MMP-2/TIMP-2 complex, a monoclonal antibody against TIMP-2 and a polyclonal antiserum against MMP-2 was used (Öberg *et al*. 2000).

The serum CrossLaps™ ELISA (Rosenquist *et al*. 1998) uses two monoclonal antibodies specific for a β-aspartate form of the peptide EKAHβDGGR derived from the α1-chain of type I collagen. For the serum CrossLaps™ assay (Osteometer BioTech A/S, Herlev, Denmark) 50 µl of the serum or synovial fluid duplicates were pipetted to streptavidin-coated microtiter wells followed by 150 µl of the monoclonal antibody suspension. The absorbance was measured and the results calculated with a Victor^2 1420 multilabel counter (Wallac, Turku, Finland). The inter- and intra-assay variations for serum CrossLaps ELISA were 5.4–7.9 % and 4.7–5.9 %, respectively. Two patients were excluded from the analysis of the rheumatoid arthritis patients (IV) because of the extremely high CrossLaps value (33939 in serum and 17815 in synovial fluid and 32423 pM in serum, 1264344 in synovial fluid), which were very likely due to a false-positive reaction via rheumatoid factor. Their RF values were 1869 and 740 kIU/l (reference value 15 kIU/l).

In the study of rheumatoid arthritis (IV), the µg/L values of the ICTP, PINP and PIIINP were changed to picomolar concentrations in order to compare the concentrations of these markers with the picomolar concentrations given by the CrossLaps assay. The µg/L values of ICTP were divided by the molecular weight 10983 g/mol and PINP by 35 000 g/mol and PIIINP by 42 000 g/mol.

4.7 Patients (III, IV)

The patients for the irradiation study (III) included 18 women treated by local radiotherapy after ablation (five cases) or resection (13 cases) for breast carcinoma at the Department of Oncology and Radiotherapy, Oulu University Hospital, during the years 1990–1997. From symmetrical sites of the irradiated and contralateral breasts of each patient, 4-mm punch biopsies of the skin were taken under local anaesthesia. The biopsies were immediately frozen and stored at -20°C until analyzed. The study was carried out in accordance with the provisions of the declaration of Helsinki after permission from the local ethical committee. Informed consent was obtained from each patient.

Forty-one patients (IV) with rheumatoid arthritis (classified as described by Arnett *et al*. 1988) from the Oulu University Hospital and the Satalinna Hospital were included in the study. The radiological state of the knee joint was assessed by one of the authors (RL), using Larsen´s method (Larsen *et al*. 1977) to compare posteroanterior radiographs with a standard series. Routine laboratory tests for the assessment of the disease activity, i.e. erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), rheumatoid factor (RF) etc. were examined from the serum. The serum and synovial fluid samples were collected in sterile tubes and stored at -20°C. The synovial fluid samples were centrifuged within one hour at 1800 g for 20 min before storage. We had a permission from the ethical committee also for this study.

4.8 Animals (II)

The bovine assay was evaluated with a trial (II) including 37 Brown Swiss cows, Simmental-Red Holstein crossbred cows, and Holstein cows (II). Cows were assigned to two groups: group 1 included 18 cows with signs of periparturient paresis (recumbence, reduced or no rumen activity, reduced or no appetite, disturbed alertness, dry nose, and abnormal heart rate), and group 2 consisted of 19 cows with a normal periparturient period (control). Blood and urine samples were taken after parturition (day 1, day of parturition) before infusion of Ca (500 ml of Calcamyl 40 MP, 3.1 g of Ca/100 ml, Dr. E. Gräub AG, Bern, Switzerland) in group 1. In group 2 the first-day sample was taken within 8 h after parturition, and on days 2,3,4,5,9, and 14 in both groups. Urine samples were analyzed for Dpd, HYP, and creatinine. Serum samples were analyzed for Ca, Mg, P, ICTP and PIIINP.

4.9 Statistical analysis of data (III, IV)

The data of the rheumatoid arthritis patients were recorded and Spearman's rank correlation coefficient tests calculated on a PC using SPSS statistical software.

The statistical differences between the concentrations of the analytes in the irradiated and contralateral skin biopsies in the case of irradiation fibrosis were tested with the t-test for paired samples or the non-parametric sign test for paired samples using SPSS statistical software.

4.10 Enzymatic degradation of the purified peptides

200 µg of trypsin-derived, purified human ICTP from bone, skin and myoma (100 µM) and collagenase-derived ICTP from bone was incubated with recombinant human cathepsin K (10 µM) in 100 mM sodium acetate/ 5 mM EDTA/5 mM cysteine buffer (pH 5.5) for 12 h at 37°C. Recombinant human cathepsin K was produced in baculo-virus-infected SF21 cells as described previously (Bossard *et al*. 1996).

Chymotrypsin digestions of type I collagen were performed in 0.2 mol/l ammonium carbonate and matrix metalloproteinase (MMP-1, -9 and -13) digestions in 50 mmol/l Tris-HCl and 10 mmol/l $CaCl_2$ (pH 7.5). The MMPs were first activated in 1 mmol/l p-aminophenylmercuric acetate for 2 h at 37°C**.** Similar control preparations without added enzyme were made contemporarily from every sample.

4.11 Analytical methods

The skin biopsies (III) were weighed, minced with a scalpel and suspended in 1 ml of phosphate-buffered saline containing 0.04% (w/w) Tween 20 (PBS-Tween), keeping the samples on an ice bath all the time. The homogenized biopsies were centrifuged at 2000 x g for 30 min at +4°C (Beckman CS-6KR). The supernatants were separated and analyzed for the concentrations of PINP, SP4, ICTP, TIMP-1, TIMP-2 and the MMP-2/TIMP-2 complex. The results were expressed as the amount of extracted antigen/mg of wet weight of the biopsy.

The insoluble tissue residue was lyophilized, suspended in 1 ml of 0.2 M $(NH_4)\ 2CO_3$, heat-denatured at +70°C for 30 min and digested with trypsin (100 µg; TPCK-treated; Worthington biochemicals, Freehold, NJ) at 37°C. This denaturation and digestion cycle was repeated twice (digestion times being 6,12 and 6 h). After the final digestion, the residual trypsin activity was destroyed by additional heating at +70°C for 30 min. The minor insoluble residue was removed by centrifugation (2000 x g for 30 min at +4°C) and the supernatants were stored at -20°C. We have previously purified the PA- and HHL-cross-linked variants of the carboxyterminal telopeptides of type I collagen and characterized them by slab gel electrophoresis and N-terminal sequence analysis. For assessing the concentrations of these two telopeptide variants, 300-µl samples of the

supernatants were separated on a pH stable C_8 HPLC column (Vydac, Hesperia, CA), and each fraction was then analyzed with the SP4 and ICTP immunoassays. The fractions containing the HHL- and PA-cross-linked telopeptides were pooled separately from each run, lyophilized and dissolved in 2 mL of PBS-tween. Then, the concentrations of the two-telopeptide variants were analyzed in the pools containing only one variant with the SP4 and ICTP assays. These results were expressed per total amount of collagens in the insoluble matrix, which was calculated from the total content of hydroxyproline (Kivirikko *et al*. 1967), assuming that it accounts for 12.4 % (w/w) of the total amino acids of all the collagen's proteins.

2.5 ml of serum and synovial fluid samples (IV) were loaded on a Sephacryl S-100 column (dimensions: height 116 cm, inner diameter 1.7 cm) equilibrated in room temperature with phosphate buffered saline (PBS) containing 0.04 % Tween-20. 20-min fractions were collected with a flow rate of 5.4 ml/hour. The concentrations of ICTP and CrossLaps were measured in each fraction after 10 times concentration by lyophilization. The serum for gel filtration was from a 55-year-old woman with rheumatoid arthritis (ICTP 6.8 µg/L, CrossLaps 9000 pM in serum). The synovial fluid for gel filtration was from a 54-years-old woman with rheumatoid arthritis (synovial fluid ICTP 9.2 µg/L, synovial fluid CrossLaps 11 700 pM). The possibility of false reaction via RF was studied by ammonium sulphate precipitation and gel filtration. The quantitative RF was measured immunoturbidimetrically by Hitachi 911 analyzer using reagents supplied by Orion Dignostica (Espoo, Finland).

The ICTP and CrossLaps assays were compared (Figure 4 and 5) after trypsin and cathepsin K digestions. After digestion of the pufied bone, skin and myoma ICTPs the ICTP and CrossLaps assays were performed on several known concentrations of the peptides.

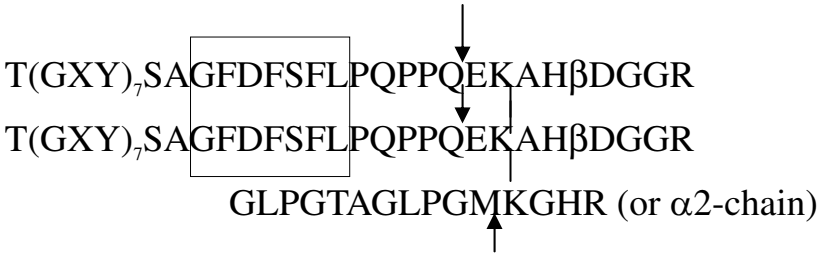
5 Results

5.1 Purification and characterization of the carboxyterminal telopeptides of type I collagen (I, II, III, additional data)

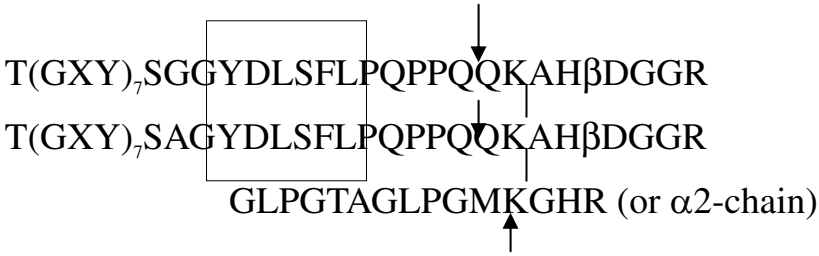
Several variants of carboxyterminal telopeptides of type I collagen were purified during the course of this study. They are: trivalent ICTP (human and bovine), divalent and non-cross-linked forms of type I collagen carboxyterminal telopeptide (human) and the HHL- and PA-cross-linked peptides from skin (human). These peptides were also purified after digestions with several enzymes cleaving at different sequences.

The peptides were characterized by amino acid analysis and N-terminal sequencing. Based on this characterization and previously published sequences from literature we conclude them to have the following sequences (cathepsin K cleavage sites indicated by arrows and epitope of the ICTP assay by a box):

Trivalently cross-linked ICTP from human bone and PA-cross-linked ICTP from human skin



Trivalently cross-linked ICTP from bovine bone



HHL-cross-linked ICTP from human skin

Synthetic peptide SP4

SAGFDGSGLPQPPQEY

When purifying the peptides, only one immunoreactive peak was detected in the ICTP assay, representing the trivalently cross-linked forms of the telopeptide, while the SP4 assay also showed reaction with the divalently cross-linked and non-cross-linked forms of the telopeptide and with HHL-cross-linked peptide (I, III). The bovine ICTP ran slightly

slower on SDS-PAGE than did the human ICTP. This does not necessarily indicate a smaller molecular mass, however; since the ICTP peptides are complex structures containing three polypeptide chains joint by a covalent cross-link, the true molecular masses cannot be reliably deduced from the molecular weight standards derived from the fractionation of myoglobin. However, the molecular masses for the two forms of trypsin-generated ICTP, differing with respect to their helical components, can be calculated from the known amino acid sequences of the constituent chains. They are 10353 for the human ICTP form $\alpha 1_C \alpha 1_C \alpha 1_H$ and 10421 for the form $\alpha 1_C \alpha 1_C \alpha 2_H$. The molecular masses of the corresponding bovine structures are 10310 and 10194. The true molecular weights of the antigens determined by MALDI-TOF analysis were: trypsin derived ICTP antigen from bone 10240 with minor component 8549, from skin only minor component 8568 and from myoma the molecular weight varied between 10416–10525, and the lower mass component 8602–8723. The sizes of the peptides in SDS-PAGE, however, indicate that the lower mass component is a degradation product during the MALDI treatment. Smaller fragments were found in MALDI analysis and these correspond to the calculated value of the two telopeptides without the helical chain (8657 g/mol).

When the cross-links are considered, most of the pyridinoline fluorescence co-eluted with ICTP, whereas the majority of the pyrrole reaction, representing another major trivalent collagen cross-link, was found in other fractions related to the aminoterminal cross-link sites (I).

From skin, two immunoreactive peaks could be separated by the pH-stable reverse phase chromatography, the form reacting better in the ICTP radioimmunoassay being much more hydrophobic. The divalent forms of the carboxyterminal telopeptide were separated after the S-100 gel filtration, so they are not present any more in this phase. In the low pH reverse phase HPLC column chromatography the peptides were further purified separately from each other. Two different immunoreactive peptides were found, the first one reacting much more weakly in the ICTP and better in the SP4 radioimmunoassay, although the majority of the absorbance at 280 nm was found with the first peak.

The data from amino acid analysis and sequencing were sufficient to identify the HHL-cross-linked carboxyterminal telopeptide of type I collagen. The second peptide was observed to resemble the trivalently cross-linked telopeptide of type I collagen found in bone, and it also reacted similarly in the ICTP radioimmunoassay, which needs two phenylalanine rich regions of the two telopeptide chains for its immunoreaction. However, no pyridinoline fluorescence was found with this peptide, so we call it the pyridinoline analogue (PA)-cross-linked peptide. In SDS-PAGE the HHL-cross-linked peptide seemed to be slightly smaller than the PA-cross-linked peptide (data not shown).

No tritiated SP4-reactive peaks were found in skin, indicating that the amount of divalently cross-linked, reducible cross-links (dehydrolysinonorleucine LNL, dehydrohydroxylysinonorleucine HLNL, dehydrodihydroxylysinonorleucine DHLNL) is small.

5.2 Radioimmunoassay for bovine ICTP (II)

Bovine ICTP assay (I) parallellism existed between bovine samples and the bovine standard. There was clearly more inhibition in the foetal bone serum than in the newborn bovine and adult bovine sera. No crossreaction with human samples could be shown.

5.3 Characterization of the enzyme cleavage sites in ICTP (I)

The following two sequences were the most abundant after purification of the cathepsin K cleaved ICTP: E-?-A-H-D-G-G-R and L-P-Q-P-P-Q-E-?-. Both sequences can be found in the carboxyterminal telopeptide of the α1 chain. Thus the cleavage by cathepsin K had occurred between F (phenylalanine) and L (leucine) as well as between Q (glutamine) and E (glutamic acid). The first step in the sequencer run also contained relatively large amounts of methionine and phenylalanine, indicating that the main cleavage site within the helical part of the cross-linked ICTP peptide is located one amino acid to the N-terminal direction from the cross-linking lysine residue.

Chymotrypsin hydrolysis of the human and bovine ICTP peptides produced, as expected, truncated ICTP fragments. The aminoterminal sequence analysis of the human ICTP peptide revealed that chymotrypsin had cleaved within the hydrophobic region located aminoterminally from the cross-link site, most frequently after the second phenylalanine residue. In the case of the bovine ICTP, chymotrypsin cleaved after the tyrosine residue in the same region between the triple-helical domain and the cross-link site.

5.4 Characterization and enzyme stability of the antigenic determinants of ICTP (I)

Polyclonal antibodies raised against human or bovine ICTP effectively bound the respective, iodinated antigens. The antibodies to bovine ICTP bound poorly to human ICTP (about 16 fold less compared to the bovine species) and antibodies to human ICTP behaved similarly with bovine ICTP. Accordingly, bovine ICTP exhibited poor immunodetection when using the assay for human ICTP and *vice versa*. Chymotrypsin hydrolysis of either bovine or human ICTP demonstrably reduced their antibody binding in the respective assays. Cleavage after the first phenylalanine residue effectively destroyed any ICTP detection in the immunoassay.

Detection of the telopeptide was between 50-150 fold lower when the ICTP contained only one intact carboxyterminal telopeptide of the α1 chain of type I collagen, with little difference between the structures containing just the linear telopeptide sequence and that

with an additional short triple-helical sequence attached. A similar phenomenon was observed with the HHL-cross-linked carboxyterminal telopeptide, which contains only one telopeptide chain.

As with chymotrypsin treatment, cathepsin K cleavage of ICTP produced a species that was no longer recognized by the immunoassay and about 100 times higher concentration of the cathepsin K -cleaved peptide was needed to give the same immunoreaction in the ICTP assay. This loss of antigenicity was completely blocked by 1 mM E-64, a potent inhibitor of cathepsin K. MMP-1, MMP-9 and MMP-13 had no effect on the ICTP immunoreaction.

5.5 The specificity and enzyme stability of the CrossLaps assay – comparison with ICTP (IV, additional data)

CrossLaps is able to react comparably with carboxyterminal telopeptides from bone, skin and leiomyoma whether digested with cathepsin K or not (Figure 4). The main differences between the CrossLaps and ICTP assays are:

1. ICTP antibodies are not able to react with the cathepsin K product, but the cathepsin K treatment does not affect the CrossLaps assay
2. ICTP works in ten times smaller concentrations than CrossLaps (Figure 5) and
3. CrossLaps assay has a remarkable hook effect in high peptide concentrations.

The cross-linking profile of the peptide seemed not to have any effect on the assay behaviour.

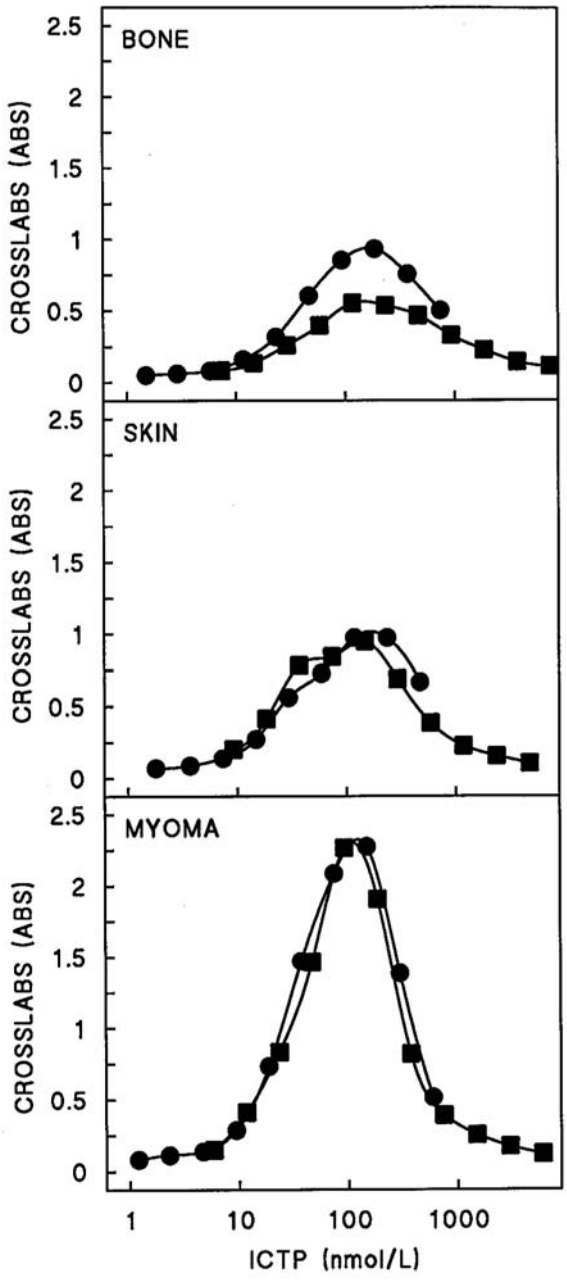


Fig. 4. The behaviour of the CrossLaps assay in different concentrations of the carboxyterminal telopeptide after trypsin (squares) of cathepsin K (circles) treatments.

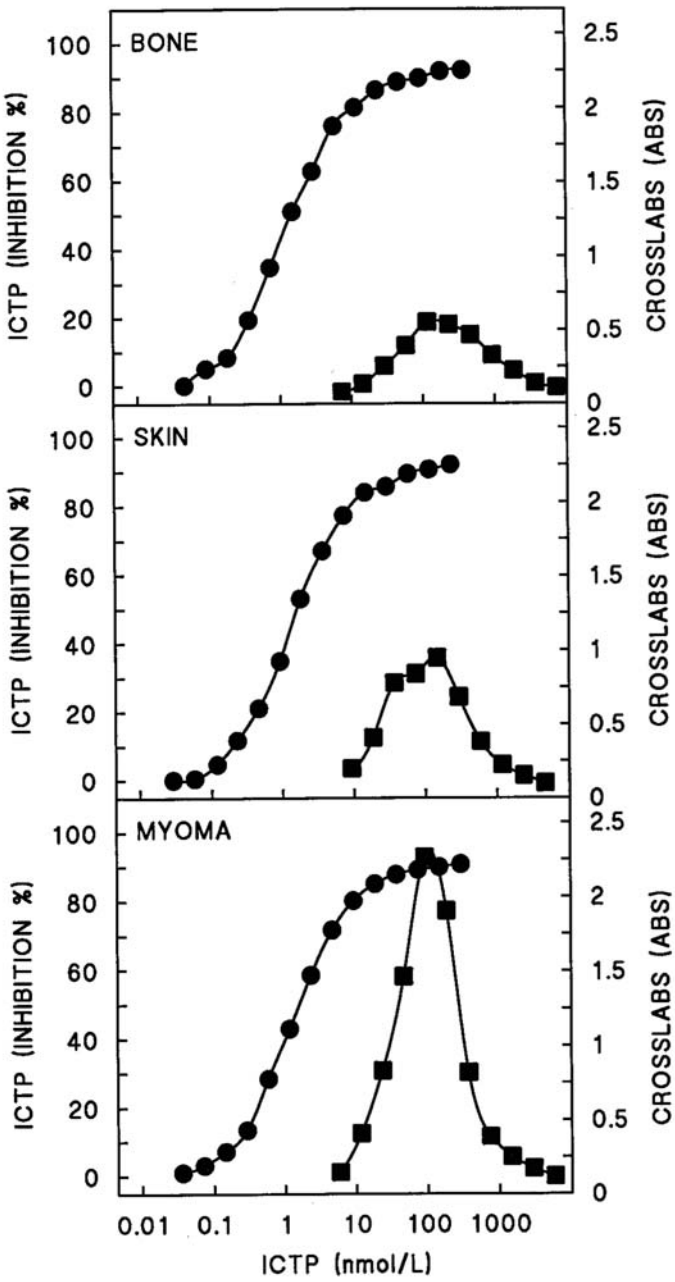


Fig. 5. The behaviour of the ICTP and CrossLaps assays in different concentrations of the trypsin-derived carbosyterminal telopeptide (nmol/L). ICTP assay is indicated as solid circles and CrossLaps as solid squares.

5.6 Irradiation fibrosis (III)

The PINP, SP4 and CrossLaps™ in the skin extracts were all significantly higher in the extract fluid of the irradiated side of the patient compared to the control side. On the other hand, knowing the poor immunoreactivity of the HHL-peptide in the ICTP assay, the ICTP assay expectedly did not detect increased collagen degradation ($p = 0.14$).

The TIMP-1 and TIMP-2 values showed no significant difference between the irradiated and control sides. In contrast to this, the MMP2/TIMP2-complex was significantly higher at the control side.

SP4, CrossLaps and PINP correlated with each other, while their correlations with ICTP were far less clear. Both ICTP and SP4, measured without the HPLC separation, were significantly higher in the digestions of the irradiated skin when compared to the control side. When the different chain combinations of the carboxyterminal telopeptide in the digestions were separated, the peptides containing only one telopeptide chain, mainly the HHL-cross-linked peptides, were shown to be the major cross-linked peptides in the skin biopsies. No pyridinoline fluorescence was found during the runs. The amount of HHL-cross-linked peptides, pooled earlier to the pyridinoline analogue-cross-linked peptides, was significantly higher on the irradiated side when corrected either by hydroxyproline determined collagen content or by dry collagen weight. No significant correlation was found when the amount of the pyridinoline analogue-cross-linked peptide was studied. Moreover, the amount of PA-cross-linked peptides was much lower when compared to the HHL-cross-linked peptides in these samples.

5.7 Evaluation of ICTP and CrossLaps in rheumatoid arthritis and analysis of the serum and synovial fluid C-terminal degradation fragments (IV)

The gel filtration analysis of serum from an RA patient shows that the main antigenic forms detected by the ICTP and CrossLaps assays are clearly separate in size. The ICTP assay does not cross-react at all with the CrossLaps antigen, whereas the CrossLaps assay reacted to some extent with the ICTP antigen. However, the serum concentration of the ICTP antigen was mostly well below the detection limit of the CrossLaps assay. In synovial fluid both antigens were partially larger in size than in serum.

Increased concentrations of markers in RA were found in 46% of the patients in the case of ICTP, 5.4% in the case of CrossLaps, 5.6% in the case of PINP and 47.2% in the case of PIIINP. The picomolar amounts were also calculated. The molar amount of ICTP was $20.8 \pm 15.2\%$ (mean±SD) of the amount of CrossLaps, whereas in synovial fluid the proportion was as high as $36.9 \pm 24\%$ (mean ± SD). The molar concentration of PINP in serum was about 10-fold higher than that of PIIINP, but in synovial fluid the ratio was opposite. The concentration of the ICTP was constantly higher or at least equal in synovial fluid compared to serum, while the ratio of CrossLaps varied. The SF:S ratios of PINP and especially that of PIIINP were much higher than those of the degradation markers.

ICTP, CrossLaps and PIIINP, but not PINP, correlated between serum and synovial fluid. At the serum level, the ICTP correlated with the three other markers, most significantly with PIIINP. The serum CrossLaps had the highest correlation with serum PINP, but no correlation with serum PIIINP. At the synovial fluid level, the most interesting finding was that the CrossLaps did not correlate with the other markers of collagen metabolism.

ESR and CRP, the markers reflecting systemic inflammation, correlated statistically significantly with serum and synovial fluid ICTP and PIIINP assays, but not with those of CrossLaps and PINP. In the present study the radiological Larsen's, Ritchie's articular as well as joint swelling scores of the examined knee joint did not reach significant correlations with any of the measured markers of collagen metabolism either in serum or in synovial fluid.

Two patients had extremely high CrossLaps concentrations. They had also high RF values. The ammonium sulphate precipitates of these two serum samples gave the absorbencies 2.910 and 2.905, which were twice the value of the highest standard. None of the other patients, whether seropositive or seronegative, gave values above the blank. In addition, serum and synovial fluid samples from the first of the above patients were studied by gel filtration and it was found that the high concentrations of the CrossLaps were, indeed, due to a false-positive reaction via RF. Both patients with extremely high serum CrossLaps concentrations were excluded from the comparisons.

6 Discussion

The assay for the carboxyterminal telopeptide of type I collagen (ICTP) was developed in 1993 (Risteli *et al.* 1993). It was soon proved to be a good tool in assessing various severe conditions, e.g. reflecting the bone invasion by malignomas (Blomqvist *et al.* 1996, Elomaa *et al.* 1992, Kylmälä *et al.* 1995). However, quite shortly afterwards great confusion and disappointment followed, since the assay was insensitive to changes in bone physiology. It did not change as much as urinary telopeptide markers after estrogen or bisphosphonate treatment (Hassager *et al.* 1994). Since most of the basic researchers in the bone field were mainly interested only in measuring physiological bone turnover, the ICTP assay obtained a poor reputation. Also commercial reasons were obviously involved. One of the opinion leaders in the field commented the assay as “waste of money”. Also more scientific statements can be found in the literature such as "perhaps this fragment marker not only reflects degradation of type I collagen, but also indicates collagen turnover through the possible cross-reactivity to collagen type II and collagen type III α-chains" (Bonde *et al.* 1994) and "ICTP has been disappointing with respect to diagnostic specificity and sensitivity for monitoring bone metabolism in the majority of patient groups" (Pedersen *et al.* 1998). In spite of these negative opinions the assay has been used in over 340 scientific publications, which can be found on the Internet (http://www.ncbi.nlm.nih.gov/PubMed).

After publishing the location of the epitope (I) it has still been said that "Whether the larger ICTP reactive fragments are generated during osteoclastic bone resorption or during collagen synthesis is not known. In fact, it has been suggested that the ICTP marker predominantly reflects collagen type I synthesis in bone and other tissues" (Christgau *et al.* 1998), but little by little more positive comments followed: "These data indicate that the ICTP assay measures another population of antigens, which probably are larger than the fragments detected by the CrossLaps assay" (Rosenqvist *et al.* 1998).

The main finding of this thesis is that the epitope of the ICTP assay lies in the phenylalanine-rich region, which has also earlier been shown to be the most immunoreactive region in the type I collagen molecule (Stoltz *et al.* 1973). This antigenic determinant is destroyed during osteoclastic resorption by cathepsin K. This explains the findings that the ICTP assay is not sensitive to changes in physiological bone turnover, such as those seen in postmenopause, during the use of estrogen replacement therapy

(Hassager *et al*. 1994) and bisphosphonate treatment (Garnero *et al*. 1994) or in Paget's disease of bone (Randall *et al*. 1996). The result gives a solid explanation to the above comments. However, this property of the ICTP assay, which was once regarded as a disadvantage, offers an advantage in monitoring pathological situations. This kind of situations are those in which bone and soft tissue type I collagen is rapidly destroyed by matrix metalloproteinases or other cathepsins than cathepsin K, e.g. rheumatoid arthritis (Hakala *et al*. 1993), multiple myeloma (Elomaa *et al*. 1992) or bone metastases from carcinomas (Aruga *et al*. 1997). The clinician does not have to consider the menopausal status or other changes in physiological bone turnover.

Determining of the epitope of the ICTP assay was not easy, since synthetic peptides could not be used due to the special cross-linked structure. We used the poor cross-reactivity of the human and bovine antigens in determining the epitope — where the difference in the amino acid sequence is, there must be the epitope (I). We had to purify the trivalent antigens from bovine and human bones. The result was further confirmed by the finding that chymotrypsin destroyed the antigenicity of the ICTP assay.

As already mentioned, the CrossLaps assay (Rosenqvist *et al*. 1998) is sensitive for physiological bone turnover (Christgau *et al*. 1998). This is explained by the fact that it is able to measure the cathepsin K fragment of carboxyterminal telopeptide produced by osteoclasts (EKAHβDGGR). We have shown in this thesis that the CrossLaps assay is able to measure also the large fragments of the carboxyterminal telopeptide of type I collagen, which are also measured by the ICTP assay (Figure 5). However, as shown in paper IV, the ICTP assay is superior to the CrossLaps assay in rheumatoid arthritis, where several MMPs have been shown to be the main collagen degrading enzymes (Konttinen *et al*. 2000, Yoshihara *et al*. 2000). The amount of cathepsin K derived fragment in serum is so high in every situation that the ICTP-like degradation is masked under it (IV). I emphasize that there is a difference in the sensitivities of these two assays, the ICTP assay being much more sensitive in general (technical properties) and, due to its epitope, for measuring the pathological collagen degradation (immunological properties). That is why the assays give separate peaks in serum and synovial fluid and cannot replace each other (IV). I conclude that for pathological degradation, where MMPs are the main degrading enzymes, the ICTP assay should be used. On the other hand, for physiological bone turnover, such as in monitoring the treatment of osteoporosis, the CrossLaps assay is one of the best choices (see Table 3). The pathological degradation in rheumatoid arthritis based on these assays was calculated to be 21% of the total (IV).

Table 2 demonstrates some of the data regarding the fragments of the carboxyterminal telopeptide of type I collagen produced by different collagen degrading enzymes. The interstitial collagenases and gelatinases exhibit two distinct proteolytic mechanisms: gelatinases digest the gelatinous peptide rapidly in individual steps with intermediate releases of partially processed substrate, whereas collagenases degrade the triple-helical heterotrimer by trapping it until all three alpha chains are cleaved (Ottl et al 2000). The MMP-2 may be the enzyme responsible for the varying-size degradation products described in paper IV (Lauer-Fields *et al*. 2000).

Table 2. The C-terminal degradation fragments of type I collagen produced by different enzymes.

Enzyme	Fragment
MMP-1, -8, -13 (collagenases)	1/4-collagen fragment (Gross et al. 1974)
MMP-3, -7, -10, -11 (stromelysins)	1/4-collagen fragment
MMP-2, -9 (gelatinases)	additional cleavage sites producing shorter than 1/4-collagen fragment
MT-MMP-1	1/4-collagen fragment (Ohuchi 1997)
Cathepsin K	EKAHDGGR
Cathepsin D	known to destroy the ICTP epitope (own unpublished result)
Cathepsin B	does not destroy the ICTP epitope (own unpublished result)

The established biochemical markers of collagen metabolism can be divided roughly into two groups depending on whether they mainly reflect normal bone turnover or local pathological process (Table 3). The conclusions are extrapolated from only one clinical condition, rheumatoid arthritis, but the general features can most probably be expanded to encompass other pathological conditions as well. In rheumatoid arthritis, ICTP and PIIINP are closely associated with the disease activity markers (IV) and reflect pathological process, whereas PINP and CrossLaps reflect overall physiological bone turnover. Another assay of type I collagen degradation, the NTx, belongs biochemically to the same group as CrossLaps. In rheumatoid arthritis, PYD has been suggested to derive from the local pathological process involving soft tissues (St Clair *et al*. 1998, Kameyama *et al*. 2000). Free Dpd has been shown to correlate better with the disease activity in rheumatoid arthritis than the total Dpd (Suzuki *et al*. 1998).

Table 3. Assays of collagen metabolism divided into two groups based on their clinical behaviour

Physiological turnove (cathepsin K)r	Pathological degradation (MMPs)
PINP	PIIINP
CrossLaps	ICTP
NTx	PYD
Total Dpd	Free Dpd

The other question was how the antigen structure (e.g. cross-linking and chain combinations) affects the ICTP antigenicity in addition to the enzyme effect (the size of the fragment) (I). We have also shown in this thesis that the ICTP assay is not able to measure the divalent or non-cross-linked forms of type I collagen (I). The ICTP assay is also unable to measure the HHL-cross-linked fragment of skin collagen (III), which is, in fact, trivalently cross-linked. What is common for these structures is that the antigen contains only one telopeptide chain and thus only one phenylalanine region. Thus the ICTP epitope was characterized as including two phenylalanine-rich regions of type I collagen carboxyterminal telopeptide. This property can be used when examining the cross-linking of skin collagen, after trypsin digestion and HPLC separation of the peptides (III). We noticed that HHL-cross-linking was increased in skin after irradiation, which resembles the change occurring during normal ageing (Yamauchi *et al*. 1988). The microstructure of the skin collagen fibres with their specific tilt angles was conserved in

this situation (Mechanic *et al*. 1987), because the HHL-cross-link is the predominant cross-link also in normal skin. If the amount of the two-telopeptide containing PA-cross-links had increased, it might have affected the tissue structure.

In this study, an assay for bovine ICTP was developed and evaluated. In cows (II) it seemed to correlate with Dpd results, reflecting bone turnover. However, in paper I it was also shown that chymotrypsin, and thus also cathepsin K, destroys the antigenicity of the bovine assay. High amounts in foetal calf serum suggest that bovine ICTP assay is measuring a fragment produced by matrix metalloproteinases.

7 Conclusions

1. The ICTP assay is not sensitive to physiological bone turnover, because the main enzyme for type I collagen catabolism in bones, cathepsin K, destroys its antigenic determinant.
2. The enzyme cleavage has no effect on the CrossLaps assay. The reason why CrossLaps is useless in many pathological situations is that the small amount of MMP-derived degradation products is masked by the high amounts of cathepsin K derived degradation products in serum.
3. The ICTP assay is not able to measure either the HHL-cross-linked, the divalent or non-cross-linked forms of the carboxyterminal telopeptide, since the antigenic determinant needs two phenylalanine-rich regions of two adjacent telopeptide chains. This property can be used in determining differently cross-linked forms of the carboxyterminal telopeptide e.g. in skin after and without irradiation.
4. The assay for bovine ICTP was developed and applied clinically. It resembles that of human and most propably measures the MMP-derived degradation products.

References

Aibe K, Yazawa H, Abe K, Teramura K, Kumegawa M, Kawashima H & Honda K (1996) Substrate specificity of recombinant osteoclast-specific cathepsin K from rabbits. Biol Pharm Bull 19:1026-1031.

Apone S, Lee MY & Eyre DR (1997) Osteoclast generate cross-linked collagen N-telopeptides (NTx) but not free pyridinolines when cultured on human bone. Bone 21:129-136.

Aruga A, Kolzumi M, Hotta R, Takahashi S & Ogata E (1997) Usefulness of bone metabolic markers in the diagnosis and follow-up of bone metastasis from lung cancer. Br J Cancer 76:760-764.

Atley LM, Mort JS, Lalumiere M & Eyre DR (2000) Proteolysis of human bone collagen by cathepsin K: characterization of the cleavage sites generating the cross-linked N-telopeptide neoepitope. Bone 26:241-247.

Bailey AJ & Lister D (1968) Thermally labile cross-links in native collagen. Nature 220:280-281.

Banda MJ & Werb Z (1981) Mouse macrophage elastase. Purification and characterization as a metalloproteinase. Biochem J 193:589-605.

Barber M, Bordoli RS, Elliott GJ, Fujimoto D & Scott JE (1982) The structure of pyridinolines. Biochem Biophys Res Comm 109:1041-1046.

Bartlett JD, Simmer JP, Xue J, Margolis HC & Moreno EC (1996) Molecular cloning and mRNA tissue distribution of a novel matrix metalloproteinase isolated from porcine enamel organ. Gene 183:123-128.

Berti PJ & Storer AC (1995) Alignment/phylogeny of the papain superfamily of cysteine proteases. J Mol Biol 246:273-283.

Bjarnason NH & Christiansen C (2000) Early response in biochemical markers predicts long-term response in bone mass during hormone replacement therapy in early postmenopausal women. Bone 26:561-569.

Blair HC, Kahn AJ, Crouch EC, Jeffrey JJ & Teitelbaum SL (1986) Isolated osteoclasts resorb the organic and inorganic components of bone. J Cell Biol 102:1164-1172.

Blair HC, Teitelbaum, SL, Grosso LE, Lacey DL, Tan HL, McCourt DW & Jeffreys JJ (1993) Extracellular-matrix degradation at acid pH. Avian osteoclast acid collagenase isolation and characterization. Biochem J 290:873–884.

Bonde M, Garnero P, Fledelius C, Qvist P, Delmas PD & Christiansen C (1997) Measurement of bone degradation products in serum using antibodies reactive with an isomerized form of an 8 amino acid sequence of the C-telopeptide of type I collagen. J Bone Miner Res 12:1028-1034.

Bonde M, Qvist P, Fledelius C, Riis BJ & Christiansen C (1994) Immunoassay for quantifying type I collagen degradation products in urine evaluated. Clin Chem 40:2022-2025.

Bossard MJ, Thomaszek TA, Thompson SK, Amegadzie BY, Hanning CR, Jones C, Kurdyla JT, McNulty DE, Drake FH, Gowen M & Levy MA (1996) Proteolytic activity of human osteoclast cathepsin K. Expression, purification, activation and substrate identification. J Biol Chem 271:12517-12524.

Blonqvist C, Risteli L, Risteli J, Virkkunen P, Sarna S & Elomaa I (1996) Markers of type I collagen degradation and synthesis in the monitoring of treatment response in bone metastases from breast carcinoma. Brit J Cancer 73:1074-1079.

Breathnach R, Matrisian LM, Gesnel MC, Staub A & Leroy P (1987) Sequences coding for part of oncogene-induced transin are highly conserved in a related rat gene. Nucleic Acids Res 15:1139-1151.

Brömme D & Okamoto K (1995) Human cathepsin O2, a novel cysteine protease highly expressed in osteoclastomas and ovary: Molecular cloning, sequencing and tissue distribution. Biol Chem Hoppe-Seyler 376:379-384.

Brömme D, Okamoto K, Wang B & Biroc S (1996) Human cathepsin O2, a matrix protein-degrading cysteine protease expressed in osteoclasts. J Biol Chem 271:2126-2132.

Burjanadze TV (2000) New analysis of the phylogenetic change of collagen thermostability. Biopolymers 53:523-528.

Busiek DF, Ross FP, McDonnell S, Murphy G, Matrisian LM & Welgus HG (1992) The matrix metalloprotease matrilysin (PUMP) is expressed in developing human mononuclear phagocytes. J Biol Chem 267:9087-9092.

Calvo MS, Eyre DR & Gundberg CM (1996) Molecular basis and clinical application of biological markers of bone turnover. Endocrine Rev 17:333-368.

Chapman HA, Riese RJ & Shi GP (1997) Emerging roles for cysteine proteinases in human biology. Annu Rev Physiol 59:63-88.

Cheung DT, DiCesare P, Benya PD, Libaw E & Nimni ME (1983) The presence of intermolecular disulfide cross-links in type III collagen. J Biol Chem 258:7774-7778.

Chin JR, Murphy G & Werb Z (1985) Stromelysin, a connective tissue-degrading metalloendopeptidase secreted by stimulated rabbit synovial fibroblasts in parallel with collagenase. Biosynthesis, isolation, characterization and substrates. J Biol Chem 260:12367-12376.

Christgau S, Rosenqvist C, Alexandersen P, Hannover Bjarnason N, Ravn P, Fledelius C (1998) Clinical evaluation of the serum CrossLaps One Step ELISA, a new assay measuring the serum concentration of bone derived degradation products of type I collagen C-telopeptides. Clin Chem 44:2290-2300.

Chu ML, de Wet W, Bernard M, Ding JF, Morabito M, Myers J, Williams C & Ramirez F (1984) Human pro alpha 1(I) collagen gene structure reveals evolutionary concervation of a pattern of introns and exons. Nature 310:337-340.

Cole AA, Chubinskaya S, Schumacher M, Huch K, Cs-Szabo G, Yao J, Mikecz K, Hasty KA & Kuettner KE (1996) Chondrocyte matrix metalloproteinase-8. Human articular chondrocytes express neutrophil collagenase. J Biol Chem 271:11023-11026.

Collier IE, Bruns GA, Goldberg GI & Gerhard DS (1991) On the structure and chromosome location of the 72- and 92-kDa human type IV collagenase genes. Genomics 9:429-434.

Colwell A & Eastell R (1996) Renal clearance of free and conjugated pyridinium crosslinks of collagen. J Bone Miner Res 11:1976-1980.

Clark IM & Murphy G (1999) Matrix proteinases. In: Dynamics of bone and cartilage metabolism. Academic Press. 672s.

Crazer R (2000) Extracellular matrix (ECM) components in a very primitive multicellular animal, the dicyemid mesozoan Kantharella antarctica. Anat Rec 259:52-59.

Curran S & Murray GI (1999) Matrix metalloproteinases in tumour invasion and metastasis. J Pathol 189:300-308.

Delaisse JM, Boyde A, Maconnachie E, Ali NN, Sear CHJ, Eeckhout Y, Vaes G & Jones SJ (1987) The effects of inhibitors of cysteine-proteinases and collagenase on the resorptive activity of isolated osteoclasts. Bone 8:305-313.

Delaisse JM, Ledent P & Vaes G (1991) Collagenolytic cysteine proteinases of bone tissue. Cathepsin B, (pro)cathepsin L and a cathepsin L-like 70 kDa proteinase. Biochem J 279:167-174.

Delaisse JM, Eeckhout Y & Vaes G (1984) In vivo and in vitro evidence for the involvement of cysteine proteinas in bone resorption. Biochem Biophys Res Commun 125:433-440.

Delaisse J-M, Engsig MT, Everts V, Ovejero MC, Ferreras M, Lund L, Vu TH, Werb Z, Winding B, Lochter A, Karsdal MA, Troen T, Kirkegaard T, Lenhard T, Heegaard A-M, Neff L, Baron R & Foged NT (2000) Proteinases in bone resorption: obvious and less obvious roles. Clin Chim Acta 291:223-234.

Dodds RA, Connor JR, Drake F, Field J & Gowen M (1998) Cathepsin K mRNA detection is restricted to osteoclasts during fetal mouse development. J Bone Miner Res 13:673-682.

Drake FH, Dodds RA, James IE, Connor JR, Debouck C, Richardson S, Lee-Rykaczewski E, Coleman L, Rieman D, Barthlow R, Hastings G & Gowen M (1996) Cathepsin K, but not cathepsins B, L, or S, is abundantly expressed in human osteoclasts. J Biol Chem 271:12511-12516.

Drake F, James I, Connor J, Rieman D, Dodds R, McCabe F, Bertolini D, Barthlow R, Hastings G & Gowen M (1994) J Bone Miner Res 9, S177.

Eastell R, Colwell A, Hapton L & Reeve J (1997) Biochemical markers of bone resorption compared with estimates of bone resorption from radiotracer kinetic studies in osteoporosis. J Bone Miner Res 12:59-65.

Elomaa I, Virkkunen P, Risteli L & Risteli J (1992) Serum concentration of the cross-linked carboxyterminal telopeptide of type I collagen (ICTP) is a useful prognostic indicator in multiple myeloma. Br J Cancer 66:337-341.

Engel J & Prockop DJ (1991) The zipper-like folding of collagen triple helices and the effects of mutations that disrupt the zipper. Annu Rev Biophys Chem 20:137-152.

Everts V, Saftig P, Korper W, Delaisse JM, Brömme D & Beertsen W (1999) Cathepsin K is more essential for long bone resorption than for resorption of calvarial bone. J Bone Miner Res 14:S356.

Exposito JY, D'Alessio M, Solursh M & Ramirez F (1992) Sea urchin collagen evolutionarily homologous to vertebrate pro-alpha 2(I) collagen. J Biol Chem 267:15559-15562.

Eyre D (1987) Collagen cross-linking amino acids. Meth Enzymol 144:115-139.

Eyre DR, Paz MA & Gallop PM (1984) Cross-linking in collagen and elastin. Ann Rev Biochem 53:717-748.

Fields C (1988) Domain organization and intron positions in Caenorhabditis elegans collagen genes: the 54-bp module hypothesis revisited. J Mol Evol 28:55-63.

Fledelius C, Johnsen AH, Cloos PAC, Bonde M & Qvist P (1997) Characterization of urinary degradation products derived from type I collagen. Identification of a β-isomerized asp-gly sequence within the C-terminal telopeptide (α1) region. J Biol Chem 272:9755-9763.

Freije JMP, Diez-Idra I, Balbin M, Sanchez LM, Blasco R, Tolivia J & Lopez-Otin C (1994) Molecular cloning and expression of collagenase-3, a novel human matrix metalloproteinase produced by breast carcinomas. J Biol Chem 269:16766-16773.

Fujimoto D, Akiba KI & Nakamura N (1977) Isolation and characterization of a fluorescent material in bovine achilles tendon collagen. Biochem Biophys Res Commun 76:1124-1129.

Fujimoto D, Moriguchi T, Ishida T & Hayashi H (1978) The structure of pyridinoline, a collagen crosslink. Biochem Biophys Res Commun 84:52-57.

Garnero P, Borel O, Byrjalsen I, Ferreras M, Drake FH, McQueney MS, Foged NT, Delmas PD & Delaisse JM (1998) The collagenolytic activity of cathepsin K is unique among mammalian proteinases. J Biol Chem 273:32347-32352.

Garnero P, Gineyts E, Arbault P, Christiansen C & Delmas PD (1995) Different effects of bisphosphonate and estrogen therapy on free and peptide-bound bone cross-link excretion. J Bone Miner Res 10:641-649.

Garnero P, Shih WJ, Gineyts E, Karpf DB & Delmas PD (1994) Comparison of new biochemical markers of bone turnover in Paget disease treated with pamidronate and a proposed model for the relationship between measurements of the different forms of pyridinoline cross-links. J Clin Endocrinol Metab 79:1693-1700.

Gelb BD, Shi GP, Chapman HA & Desnick RJ (1996) Pycnodysostosis, a lysosomal disease caused by cathepsin K deficiency. Science 273:1236-1238.

Goldberg GI, Wilhelm SM, Kronberger A, Bauer EA, Grant GA & Eisen AZ (1986) Human fibroblast collagenase. Complete primary structure and homology to an oncogene transformation-induced rat protein. J Biol Chem 261:6600-6605.

Gowen M, Lazner F, Dodds R, Kapadia R, Field J, Tavaria M, Bertoncello I, Drake F, Zavarselk S, Tellis I, Hertzok P, Debouck C & Kola I (1999) Cathepsin K knockout mice develop osteopetrosis due to a deficit in matrix degradation but not demineralization. J Bone Miner Res 14:1654-1663.

Gross J, Harper E, Harris ED, McCroskery PA, Highberger JH, Corbett C & Kang AH (1974) Animal collagenases: specificity of action, and structures of the substrate cleavage site. Biochem Biophys Res Commun 61: 605-612.

Hakala M, Risteli J, Manelius J, Nieminen P & Risteli J (1993) Increased type I collagen degradation correlates with disease severity in rheumatoid arthritis. Ann Rheum Dis 52:866-869.

Hanemaaijer R, Sorsa T, Konttinen YT, Ding Y, Sutinen M, Visser H, Van Hinsbergh VWH, Helaakoski T, Kainulainen T, Rönkä H, Tschesche H & Salo T (1997) Matrix metalloproteinase-8 is expressed in rheumatoid synovial fibroblasts and endothelial cells. J Biol Chem 272:31504-31509.

Hanson DA & Eyre DR (1996) Molecular site specificity of pyridinoline and pyrrole cross-links in type I collagen of human bone. J Biol Chem 271:26508-26516.

Hanson DA, Weis MAE, Bollen AM, Maslan SL, Singer FR & Eyre DR (1992) A specific immunoassay for monitoring human bone resorption: Quantitation of type I collagen cross-linked N-telopeptides in urine. J Bone Miner Res 7:1251-1258.

Harris ED & Krane SM (1974) Collagenases (first of three parts). N Engl J Med 291:557-563.

Harris ED & Krane SM (1974) Collagenases (second of three parts). N Engl J Med 291:605-609.

Harris ED & Krane SM (1974) Collagenases (third of three parts). N Engl J Med 291:652-661.

Hassager C, Jensen LT, Pødenphant J, Thomsen K & Christiansen C (1994) The carboxy-terminal pyridinoline cross-linked telopeptide of type I collagen in serum as a marker of bone resorption: the effect of nandrolone decanoate and hormone replacement therapy. Calcif Tissue Int 54:30-33.

Hassager C, Risteli J, Manelius J, Nieminen P & Risteli J (1994) Effect of the menopause and hormone replacement therapy on the carboxy-terminal pyridinoline cross-linked telopeptide of type I collagen. Osteopor Int 4:349-352.

Helseth DL & Veis A (1984) Cathepsin D-mediated processing of procollagen: Lysosomal enzyme involvement in secretory processing of procollagen. Proc Natl Acad Sci USA 81:3302-3306.

Henkel W, Glanville RW & Greifendorf D (1987) Characterization of a type I collagen trimeric cross-linked peptide from calf aorta and its cross-linked structure. Eur J Biochem 165:427-436.

Hidalgo M & Ekchardt SG (2001) Development of matrix metalloproteinase inhibitors in cancer therapy. J Nat Cancer Inst 93:178-193.

Hideaki N & Woessner JF (1999) Matrix metalloproteinases. Minireview. J Biol Chem 274:21491-21494.

Ho N, Punturieri A, Wilkin D, Szabo J, Johnson M, Whaley J, Davis J, Clark A, Weiss S & Francomano C (1999) Mutations of CTSK result in pycnodysostosis via a reduction in cathepsin K protein. J Bone Miner Res 14:1649-1653.

Hoshi K, Kemmotsu S, Takeuchi Y, Amizuka N & Ozawa H (1999) The primary calcification in bones follows removal of decorin and fusion of collagen fibrils. J Bone Miner Res 14:273-280.

Hou WS, Bromme D, Zhao Y, Mehler E, Dushey C, Weinstein H, Miranda CS, Fraga C, Greig F, Carey J, Rimoin DL, Desnick RJ & Gelb BD (1999) Characterization of novel cathepsin K mutations in the pro and mature polypeptide regions causing pycnodysostosis. J Clin Invest 103:731-738.

Housley T, Tanzer ML, Henson E & Gallop PM (1975) Collagen cross-linking: isolation of hydroxyaldol-histidine, a naturally-occurring crosslink. Biochem Biophys Res Commun 67:824-830.

Hughes AL (1994) Evolution of cysteine proteinases in eukaryotes. Mol Phylogenet Evol 3:310-321.

Inaoka T, Bilbe G, Ishibashi O, Tezuka K, Kumegawa M & Kokubo T (1995) Molecular cloning of human cDNA for cathepsin K: Novel cysteine proteinase predominantly expressed in bone. Biochem Biophys Res Commun 206: 89- 96.

Inui T, Ishibashi O, Inaoka T, Origane Y, Kumegawa M, Kokubo T & Yamamura T (1997) Cathepsin K antisense oligodeoxynucleotide inhibits osteoclastic bone resorption. J Biol Chem 272:8109-8112.

Johansson N, Airola K, Grenman R, Kariniemi AL, Saarialho-Kere U & Kähäri VM (1997a) Expression of collagenase-3 (matrix metalloproteinase-13) in squamous cell carcinomas of the head and neck. Am J Pathol 151:499-508.

Johansson N, Saarialho-Kere U, Airola K, Herva R, Nissinen L, Westermarck J, Vuorio E, Heino J & Kähäri VM (1997b) Collagenase-3 (MMP-13) is expressed by hypertrophic chondrocytes, periosteal cells, and osteoblasts during human fetal bone development. Dev Dyn 208:387-397.

Johansson N, Westermarck J, Leppä S, Häkkinen L, Koivisto L, Lopez-Otin C, Peltonen J, Heino J & Kähäri VM (1997c) Collagenase 3 (matrix metalloproteinase 13) gene expression by HaCaT keratinocytes is enhanced by tumor necrosis factor alpha and transforming growth factor beta. Cell Growth Differ 8:243-250.

Johnson M, Polymeropoulos M, Vos H, de Ortiz Luna R & Francomano C (1996) A nonsense mutation in the cathepsin K gene observed in a family with pycnodysostosis. Genome Res 6:1050-1055.

Jukkola A, Tähtelä R, Tholix E, Vuorinen K, Blanco G, Risteli L & Risteli J (1997) Aggressive breast cancer leads to discrepant serum levels of the type I procollagen propeptides PINP and PICP. Cancer Res 57:5517-5520.

Kadler KE, Holmes DF, Trotter JA & Chapman JA (1996) Collagen fibril formation. Review article. Biochem J 361: 1-11.

Kadler KE (1995) Type I collagen (Collagen I). In Protein Profile, Volume 2, Extracellular Matrix 1, pages 524-535. Academic Press

Kafienah W, Brömme D, Buttle DJ, Croucher LJ & Hollander AP (1998) Human cathepsin K cleaves native type I and II collagens at the N-terminal end of the triple helix. Biochem J 331:727-732.

Kameyama O, Nakahigashi Y, Nakao H, Uejima D & Tsuji H (2000) Activity of rheumatoid arthritis and urinary pyridinoline and deoxypyridinoline. J Orthop Sci 5:385-389.

Kamiya T, Kobayashi Y, Kanaoka K, Nakashima T, Kato Y, Mizuno A, & Sakai H (1998) Fluorescence microscopic demonstration of cathepsin K activity as the major lysosomal cysteine proteinase in osteoclasts. J Biochem 123: 752-759.

Karelina TV, Golberg GI & Eisen AZ (1994) Matrilysin (PUMP) correlates with dermal invasion during appendageal development and cutaneous neoplasia. J Invest Dermatol 103:482-487.

Kimura S (1983) Vertebrate skin type I collagen: comparison of bony fishes with lamprey and calf. Comp Biochem Physiol B 74:525-528.

Kivirikko K & Myllylä R (1983) Kollageenien geeniperhe. Duodecim 99:1373-1382.

Kivirikko KE, Laitinen O & Prockop DJ (1967) Modifications of a specific assay for hydroxyproline in urine. Anal Biochem 19:249-255.

Konttinen YT, Li TF, Mandelin J, Liljeström M, Sorsa T, Santavirta S & Virtanen I (2000) Increased expression of extracellular matrix metalloproteinase inducer in rheumatoid arthritis. Arthritis Rheum 43:275-280.

Kuboki Y, Tsuzaki M, Sasaki S, Liu CF & Mechanic GL (1981) Location of the intermolecular cross-links in bovine dentin collagen, solubilization with trypsin and isolation of cross-link peptides containing dihydrolysinonorleucine and pyridinoline. Biochem Biophys Res Commun 102:119-126.

Kuypers R, Tyler M, Kurth LB, Jenkins ID & Horgan D (1992) Identification of the loci of the collagen associated Ehrlich chromogen in type I collagen confirms its role as a trivalent cross-link. Biochem J 283:129-136.

Kylmälä T, Tammela TLJ, Risteli L, Risteli J, Kontturi M & Elomaa I (1995) Type I collagen degradation product (ICTP) gives information about the nature of bone metastases and has prognostic value in prostate cancer. Brit J Cancer 71:1061-1064.

Kähäri VM & Saarialho-Kere U (1997) Matrix metalloproteinases in skin. Exp Dermatol 6:199-213.

LaLonde JM, Zhao B, Janson JA, D'Alessio KJ, McQueney MS, Orsini MJ, Debouck CM & Smith WW (1999) The crystal structure of human procathepsin K. Biochemistry 38:862-869.

Lauer-Fields JL, Tuzinski KA, Shimokawa K, Nagase H & Fields GB (2000) Hydrolysis of triple-helical collagen peptide models by matrix metalloproteinases. J Biol Chem 275:13282-13290.

Lerner UH & Grubb A (1992) Human cystatin C, a cysteine proteinase inhibitor, inhibits bone resorption in vitro stimulated by parathyroid hormone and parathyroid hormone-related peptide of malignancy. J Bone Miner Res 7:433-439.

Lindy O, Konttinen YT, Sorsa T, Ding Y, Santavirta S, Ceponis A & Lopez-Otin C (1997) Matrix metalloproteinase 13 (collagenase 3) in human rheumatoid synovium. Arthritis Rheum 40:1391-1399.

Lefebvre O, Wolf C, Limacher JM, Hutin P, Wendling C, LeMeur M, Basset P & Rio MC (1992) The breast cancer-associated stromelysin-3 gene is expressed during mouse mammary gland apoptosis. J Cell Biol 119:997-1002.

Li YP, Alexander M, Wucherpfennig AL, Yelick P, Chen W & Stashenko P (1995) Cloning and complete coding sequence of a novel human cathepsin expressed in giant cells of osteoclastomas. J Bone Miner Res 10:1197-2202.

Littlewood-Evans AJ, Bilbe G, Bowler WB, Farley D, Wlodarski B, Kokubo T, Inaoka T, Sloane J, Evans DB & Gallagher JA (1997) The osteoclast-associated protease cathepsin K is expressed in human breast carcinoma. Cancer Res 57:5386-5390. (B)

Littlewood-Evans A, Kokubo R, Ishibashi O, Inaoka T, Wlodarski B, Gallagher JA & Bilbe G (1997) Localization of cathepsin K in human osteoclasts by in situ hybridization and immunohistochemistry. Bone 20:81-86. (A)

Llano E, Pendas AM, Freije JP, Nakano A, Knäuper V, Murphy G & Lopez-Otin C (1999) Identification and characterization of human MT5-MMP, a new membrane-bound activator of progelatinase overexpressed in brain tumors. Cancer Res 59:2570-2576.

Llano E, Pendas AM, Knäuper V, Sorsa T, Salo T, Salido E, Murphy G, Simmer JP, Bartlett JD & Lopez-Otin C (1997) Identification and structural and functional characterization of human enamelysin (MMP-20). Biochemistry 36:15101-15108.

Lohi J, Wilson CL, Roby JD & Parks WC (2001) Epilysin, a novel human matrix metalloproteinase (MMP-28) expressed in testis and keratinocytes and in response to injury. J Biol Chem 274:10134-10144.

Matrisian LM (1992) The matrix-degrading metalloproteinases. Bioessays 14:455-463.

Mechanic GL, Gatz EP, Hemni M, Noyes C & Yamauchi M (1987) Locus of a histidino-based, stable trifunctional, helix to helix collagen cross-link: stereospecific collagen structure of type I skin fibrils. Biochemistry 26:3500-3509.

Melkko J, Kauppila S, Risteli L, Haukipuro K, Jukkola A & Risteli J (1996) Immunoassay for the intact aminoterminal propeptide of human type I procollagen (PINP). Clin Chem 42:947-54.

Melkko J, Niemi S, Risteli L & Risteli J (1990) Radioimmunoassay of the carboxyterminal propeptide of human type I procollagen. Clin Chem 36:1328-1332.

Mitchell PG, Magna HA, Reeves LM, Lopresti-Morrow LL, Yocum SA, Rosner PJ, Geoghegan KF & Hambor JE (1996) Cloning, expression, and type II collagenolytic activity of matrix metalloproteinase-13 from human osteoarthritic cartilage. J Clin Invest 97:761-768.

Monboisse J-C, Bellon G, Randoux A, Dufer J & Borel J-P (1990) Activation of human neutrophils by type I collagen. Biochem J 270:459-462.

Montcourrier P, Mangeat PH, Salazar G, Morisset M, Sahuquet A & Rochefort H (1990) Cathepsin D in breast cancer cells can digest extracellular matrix in large acidic vesicles. Cancer Res 50:6045-6054.

Nagase H & Woessner JF (1999) Matrix metalloproteinases. Minireview. J Biol Chem 274:21491-21494.

Nishi Y, Atley L, Eyre DE, Edelson JG, Superti-Furga A, Yasuda T, Desnick RJ & Gelb BD (1999) Determination of bone markers in pycnodysostosis: effects of cathepsin K deficiency on bone matrix degradation. J Bone Miner Res 14:1902-1908.

Nomura H, Sato H, Seiki M, Mai M & Okada Y (1995) Expression of membrane-type matrix metalloproteinase in human gastric carcinomas. Cancer Res 55:3263-3266.

Ohuchi E, Imai K, Fujii Y, Sato H, Seiki M & Okada Y (1997) Membrane type I matrix metalloproteinase digests interstitial collagens and other extracellular matrix macromolecules. J Biol Chem 272:2446-2451.

Okada A, Bellocq JP, Rouyer N, Chenard MP, Rio MC, Chambon P & Basset P (1995) Membrane-type matrix metalloproteinase (MT-MMP) gene is expressed in stromal cells of human colon, breast, and head and neck carcinomas. Proc Natl Acad Sci U S A 92:2730-2734.

Okada Y, Naka K, Kawamura K, Matsumoto T, Nakanishi I, Fujimoto N, Sato H & Seiki M (1995) Localization of matrix metalloproteinase 9 (92-kilodalton gelatinase/type IV collagenase = gelatinase B) in osteoclasts: implications for bone resorption. Lab Invest 72:311-322.

Orgel JP, Wess TJ & Miller A (2000) The in situ conformation and axial location of the intermolecular cross-linked non-helical telopeptides of type I collagen. Structure 8:137-142.

Ottl J, Gabriel D, Murphy G, Knauper V, Tominage Y, Nagase H, Kroger M, Tschesche H, Bode W & Moroder L (2000) Recognition and catabolism of synthetic heterotrimeric collagen peptides by matrix metalloproteinases. Chem Biol 7:119-132.

Park HI, Ni J, Gerkema FE, Liu D, Belozerov VE & Sang QX (2000) Identification and characterization of human endometase (matrix metalloproteinase-26) from endometrial tumor. J Biol Chem 275:20540-20544.

Pedersen BJ, Ravn P & Bonde M (1998) Type I collagen C-telopeptide degradation products as bone resorption markers. J Clin Ligand Assay 21:118-127.

Pendas AM, Knäuper V, Puente XS, Llano E, Mattei MG, Apte S, Murphy G & Lopez-Otin C (1997) Identification and characterization of a novel human matrix metalloproteinase with unique structural characteristics, chromosomal location and tissue distribution. J Biol Chem 272:4281-4286.

Prockop DJ, Kivirikko KI, Tudermann L & Guzman NA (1979) The biosynthesis of collagen and its disorders. New Engl J Med 301: 13-23 & 77-78.

Puente XS, Pendas AM, Llano E, Velasco G & Lopez-Otin C (1996) Molecular cloning of a novel membrane-type matrix metalloproteinase from a human breast carcinoma. Cancer Res 56:944-949.

Randall AG, Kent GN, Garcia-Webb P, Bhagat CI, Pearce DJ, Gutteridge DH, Prince RL, Stewart G, Stuckey B, Will RK, Retallack RW, Price RI & Ward L (1996) Comparison of biochemical markers of bone turnover in Paget disease treated with pamidronate and a proposed model for the relationship between measurements of the different forms of pyridinoline cross-links. J Bone Miner Res 11:1176-1184.

Rantakokko J, Aro HT, Savontaus M & Vuorio E (1996) Mouse cathepsin K: cDNA cloning and predominant expression of the gene in osteoclasts, and in some hypertrophying chondrocytes during mouse development. FEBS Lett 393:307-313.

Reponen P, Sahlberg C, Munaut C, Thesleff I & Tryggvason K (1994) High expression of 92-kD type IV collagenase (gelatinase B) in the osteoclast lineage during mouse development. J Cell Biol 124:1091-1102.

Ripley C & Bienkowski RS (1997) Localization of procollagen I in the lysosome/endosome system of human fibroblasts. Exp Cell Res 236:147-154.

Risteli J, Elomaa I, Niemi S, Novamo A & Risteli L (1993) Radioimmunoassay for the pyridinoline cross-linked carboxyterminal telopeptide of type I collagen: A new serum marker of bone collagen degradation. Clin Chem 39:635-640.

Risteli J, Niemi S, Trivedi P, Mäentausta O, Mowat AP & Risteli L (1988) Rapid equilibrium radioimmunoassay for the amino-terminal propeptide of human type III procollagen. Clin Chem 34:715-718.

Robey (1995) Biochemistry of bone. In Osteoporosis, eds. Riggs & Melton.

Robins SP & Bailey AJ (1975) The chemistry of the collagen cross-links. The mechanism of stabilization of the reducible intermediate cross-links. Biochem J 149:381-385.

Robins SP, Shimokomaki M & Bailey AJ (1973) The chemistry of collagen cross-links. Age-related changes in the reducible components of intact bovine collagen fibres. Biochem J 131:771-780.

Royce PM & Barnes MJ (1985) Failure of highly purified lysyl hydroxylase to hydroxylate lysyl residues in the non-helical regions of collagen. Biochem J 230:475-480.

Rosenqvist C, Fledelius C, Christgau S, Pedersen BJ, Bonde M, Qvist P & Christiansen C (1998) Serum CrossLaps One Step ELISA; First application of monoclonal antibodies for measurement in serum of bone-related degradation products from C-terminal telopeptides of type I collagen. Clin Chem 44:2281-2289.

Rubinacci A, Melzi R, Zampino M, Soldarini A & Villa I (1999) Total and free deoxypyridinoline after acute osteoclast activity inhibition. Clin Chem 45:1510-1516.

Runnegar B (1985) Collagen gene contruction and evolution. J Mol Evol 22:141-149.

Saarialho-Kere UK, Crouch EC & Parks WC (1995) Matrix metalloproteinase matrilysin is constitutively expressed in adult human exocrine epithelium. J Invest Dermatol 105:190-196.

Saftig P, Hunziker E, Wehmeyer O, Jones S, Boyde A, Rommerskirch W, Detlev Moritz J, Schu P & von Figura K (1998) Impaired osteoclastic bone resorption leads to osteopetrosis in cathepsin K deficient mice. Proc Natl Acad Sci 95:13453-13458.

Sakurai Y, Sullivan M, & Yamada Y (1986) Alpha 1 type IV collagen gene evolved differently form fibrillar collagen genes. J Biol Chem 261:6654-6657.

Sato T, del Carmen Ovejero M, Hou P, Heegaard AM, Kumegawa M, Foged NT & Delaisse JM (1997) Identification of the membrane-type matrix metalloproteinase MT1-MMP in osteoclasts. J Cell Sci 110:589-596.

Scott JE, Hughes EW & Shuttleworth A (1981) A collagen-associated Ehrlich chromogen: a pyrrolic cross-link? Biosci Rep 1:611-618.

Scott JE, Qian R, Henkel W & Glanville RW (1983) An Ehrlich chromogen in collagen cross-links. Biochem J 209:263-264.

Shapiro SD, Griffin GL, Gilbert DJ, Jenkins NA, Copeland NG, Welgus HG, Senior RM & Ley TJ (1992) Molecular cloning, chromosomal location, and bacterial expression of a murine macrophage metalloelastase. J Biol Chem 267:4664-4671.

Shapiro SD, Kobayashi DK & Ley TJ (1993) Cloning and characterization of a unique elastolytic metalloproteinase produced by human alveolar macrophages. J Biol Chem 268:23824-23829.

Shi GP, Chapman HA, Bhairi Sm, DeLeeuw C, Reddy VY & Weiss SJ (1995) Molecular cloning of human cathepsin O, a novel endoproteinase and homologue of rabbit OC2. FEBS Lett 357: 129-134.

Siegel RC (1976) Collagen cross-linking. Synthesis of collagen cross-links in vitro with highly purified lysyl oxidase. J Biol Chem 251:5786-5792.

Sivaraman J, Lalumiere M, Menard R & Cygrel M (1999) Crystal structure of wild-type human procathepsin K. Protein Sci 8:283-290.

StClair EW, Mook SA, Wilkinson WE, Sanders L, Lang T & Greenwald RA (1998) A cross sectional analysis of 5 different markers of collagen degradation in rheumatoid arthritis. J Rheumatol 25:1472-1479.

Stoltz M, Timpl R, Furthmayr H & Kühn K (1973) Structural and immunogenic properties of a major antigenic determinant in neutral salt-extracted rat-skin collagen. Eur J Biochem 37:287-294.

Sukhova GK, Shi GP, Simon DI, Chapman HA & Libby P (1998) Expression of the elastolytic cathepsins S and K in human atheroma and regulation of their production in smooth muscle cells. J Clin Invest 102:576-583.

Suzuki M, Takahashi M, Miyamoto S, Hoshino H, Kushida K, Miura M & Inoue T (1998) The effects of menopausal status and disease activity on biochemical markers of bone metabolism in female patients with rheumatoid arthritis. Brit J Dermatol 37:653-658.

Takino T, Sato H, Shinagawa A & Seiki M (1995) Identification of the second membrane-type matrix metalloproteinase (MT-MMP-2) gene from a human placenta cDNA library. MT-MMPs form a unique membrane-type subclass in the MMP family. J Biol Chem 270:23013-23020.

Tezuka K, Nemoto K, Tezuka Y, Sato T, Ikeda Y, Kobori M, Kawashima H, Eguchi H, Hakeda Y & Kumegawa M (1994) Identification of matrix metalloproteinase 9 in rabbit osteoclasts. J Biol Chem 269:15006-15009.

Tezuka K, Sato T, Kamioka H, Niveide PJ, Tanaka K, Matsuo T, Ohta M, Kurihara N, Hakeda Y & Kumegava M (1992) Identification of osteopontin in isolated rabbit osteoclasts. Biochem Biophys Res Commun 186: 911-917.

Tilson MD, Dreyer RN, Hudson A, Cotte RJ & Tanzer ML (1985) Partial characteristics of an analog of pyridinoline isolated from human skin. Biochem Biophys Res Comm 126:1222-1227.

Thompson SK, Halbert SM, Bossard MJ, Tomaszek TA, Levy MA, Zhao B, Smith WW, Abdel-Meguid SS, Janson CA, D'Alessio KJ, McQueney MS, Amegadzie BY, Hanning CR, DesJarlais RL, Briand J, Sarkar SK, Huddleston MJ, Ijames CF, Carr SA, Garnes KT, Shu A, Heys JR, Brdbeer J, Zembryki D, Lee-Rykaczewski L, James IE, Lark MW, Drake FH, Gowen M, Gleason JG & Veber DF (1997) Design of potent and selective human cathepsin K inhibitors that span the active site. Proc Natl Acad Sci 94:14249-14254.

Uria JA & Lopez-Otin C (2000) Matrilysin-2, a new matrix metalloproteinase expressed in human tumors and showing the minimal domain organization required for secretion, latency and activity. Cancer Res 60:4745-4751.

Valtavaara M, Papponen H, Pirttilä AM, Hiltunen K, Helander H & Myllylä R (1997) Cloning and characterization of a novel human lysyl hydroxylase isoform highly expressed in pancreas and muscle. J Biol Chem 272:6831-6834.

Velasco G, Gal S, Merlos-Suarez A, Ferrando AA, Alvarez S, Nakano A, Arribas J & Lopez-Otin C (2000) Human MT6-matrix metalloproteinase: identification, progelatinase A activation, and expression in brain tumors. Cancer Res 60:877-882.

Velasco G, Pendas AM, Fueyo A, Knäuper V, Murphy G & Lopez-Otin C (1999) Cloning and characterization of human MMP-23, a new matrix metalloproteinase predominantly expressed in reproductive tissues and lacking conserved domains in other family members. J Biol Chem 274:4570-4576.

Verzar F (1964) Aging of the collagen fiber. Int Rev Connect Tissue Res 2:234-300.

Von Der Mark K (1999) Structure, biosynthesis, and gene regulation of collagens in cartilage and bone. Seibel MJ, Robins SP & Bilezikian JP (eds) Dynamics of bone and cartilage metabolism. Academic Press 1999, p 3-28.

Votta BJ, Levy MA, Badger A, Bradbeer J, Dodds RA, James IA, Thompson S, Bossard MJ, Carr T, Connor JR, Tomaszek TA, Szewczuk L, Drake FH, Veber DF & Gowen M (1997) Peptide aldehyde inhibitors of cathepsin K inhibit bone resorption both in vitro and in vivo. J Bone Miner Res 12:1396-1406.

Wardale RJ & Duance VC (1993) Quantification and immunolocalisation of porcine articular and growth plate collagens. J Cell Sci 105:975-984.

Wernicke D, Seyfert C, Hinzmann B & Gromica-Ihle E (1996) Cloning of collagenase 3 from the synovial membrane and its expression in rheumatoid arthritis and osteoarthritis. J Rheumatol 23:590-595.

Wichers M, Schmidt E, Bidlingmaier F & Klingmüller D (1999) Diurnal rhythm of CrossLaps in human serum. Clin Chem 45:1858-1860.

Wilhelm SM, Shao ZH, Housley TJ, Seperack PK, Baumann AP, Gunja-Smith Z & Woessner JF Jr (1993) Matrix metalloproteinase-3 (stromelysin-1). Identification as the cartilage acid metalloprotease and effect of pH on catalytic proterties and calcium affinity. J Biol Chem 268:21906-21913.

Will H & Hinzmann B (1995) cDNA sequence and mRNA tissue distribution of a novel human matrix metalloproteinase with a potential transmembrane segment. Eur J Biochem 231:602-608.

Wilson CL & Matrisian LM (1996) Matrilysin: an epithelial matrix metalloproteinase with potentially novel functions. Int J Biochem Cell Biol 28:123-136.

Wucherpfennig AL, Li YP, Stetler-Stevenson WG, Rosenberg AE & Stashenko P (1994) Expression of 92 kD type IV collagenase/gelatinase B in human osteoclasts. J Bone Miner Res 9:549-556.

Yamauchi M, London RE, Guenat C, Hashimoto F & Mechanic GL (1987) Structure and formation of a stable histidino-based trifunctional cross-link in skin collagen. J Biol Chem 262:11428-11434.

Yamauchi M, Woodley DT & Mechanic GL (1988) Aging and cross-linking of skin collagen. Biochem Biophys Res Comm 152:898-903.

Yamaza T, Goto T, Kamiya T, Kobayashi Y, Sakai H & Tanaka T (1998) Study of immunoelectron microscopic localization of cathepsin K in osteoclasts and other bone cells in the mouse femur. Bone 23:499-509.

Yoshihara Y, Nakamura H, Obata K, Yamada H, Hayakawa T, Fujikawa K & Okada Y (2000) Matrix metalloproteinases and tissue inhibitors of metalloproteinases in synovial fluids from patients with rheumatoid arthritis and osteoarthritis. Ann Rheum Dis 59:455-461.

Öberg Å, Höyhtyä M, Travelin B, Stenling R & Lindmark G (2000) Limited value of preoperative serum analyses of matrix metalloproteinases (MMP-2, MMP-9) and tissue inhibitors of matrix metalloproteinases (TIMP-1, TIMP-2) in colorectal cancer. Anticancer Res 20:1085-1091.