CARBOXYTERMINAL DEGRADATION PRODUCTS OF TYPE I COLLAGEN

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OULUN YLIOPISTO, OULU 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 divalent or histidinohydroxysinonorleucine (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

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Several collaborators have contributed to this study.

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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.

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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

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACP</td>
<td>aldol condensation product</td>
</tr>
<tr>
<td>AH</td>
<td>aldol histidine</td>
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<tr>
<td>AMC</td>
<td>aminomethylcoumarin</td>
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<tr>
<td>BiP</td>
<td>immunoglobulin heavy chain binding protein</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>Cbz</td>
<td>benzylxycarbonyl</td>
</tr>
<tr>
<td>cDNA</td>
<td>complement DNA</td>
</tr>
<tr>
<td>CLSPA</td>
<td>chromatographically purified collagenase</td>
</tr>
<tr>
<td>COL1A1</td>
<td>gene for the α1-chain of type I collagen</td>
</tr>
<tr>
<td>COL1A2</td>
<td>gene for the α2-chain of type I collagen</td>
</tr>
<tr>
<td>C(OOH)-terminal</td>
<td>carboxyterminal</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>DEAE</td>
<td>diethylaminoethyl</td>
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<tr>
<td>DHLN</td>
<td>dihydroxylysinoonorleucine</td>
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<tr>
<td>Dpd</td>
<td>lysyl pyridinoline</td>
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<tr>
<td>D-Pyr</td>
<td>lysyl pyridinoline</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>GHYL</td>
<td>galactosyl hydroxylysine</td>
</tr>
<tr>
<td>Gly-X-Y</td>
<td>glycine-unknown amino acid-unknown amino acid</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDS</td>
<td>Ehlers-Danlos syndrome</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetra-acetate</td>
</tr>
<tr>
<td>ESR</td>
<td>erythrocyte sedimentation rate</td>
</tr>
<tr>
<td>HHL</td>
<td>histidinohydroxylysinoonorleucine</td>
</tr>
<tr>
<td>HLN</td>
<td>hydroxylysinoonorleucine</td>
</tr>
<tr>
<td>HP</td>
<td>hydroxylysyl pyridinoline</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HRT</td>
<td>hormone replacement therapy</td>
</tr>
<tr>
<td>HSP47</td>
<td>heat shock protein 47</td>
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<tr>
<td>HYP</td>
<td>hydroxyproline</td>
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<tr>
<td>ICTP</td>
<td>carboxyterminal telopeptide of type I collagen</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>LN</td>
<td>lysinonorleucine</td>
</tr>
<tr>
<td>LP</td>
<td>lysyl pyridinoline</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>matrix assisted laser desorption ionization - time of flight mass spectrometry</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MT-MMP</td>
<td>membrane type matrix metalloproteinase</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>N-terminal</td>
<td>aminoterminal</td>
</tr>
<tr>
<td>Ntx</td>
<td>assay for the N-terminal telopeptide of type I collagen</td>
</tr>
<tr>
<td>OI</td>
<td>osteogenesis imperfecta</td>
</tr>
<tr>
<td>PA</td>
<td>pyridinoline analogue</td>
</tr>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PDI</td>
<td>protein disulphide isomerase</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PICP</td>
<td>carboxyterminal propeptide of type I collagen</td>
</tr>
<tr>
<td>PIIIINP</td>
<td>aminoterminal propeptide of type III collagen</td>
</tr>
<tr>
<td>PINP</td>
<td>aminoterminal propeptide of type I collagen</td>
</tr>
<tr>
<td>PML</td>
<td>polymorphonuclear leukocytes</td>
</tr>
<tr>
<td>PPI</td>
<td>peptidyl-prolyl cis-trans-isomerase</td>
</tr>
<tr>
<td>Pyr</td>
<td>hydroxylysyl pyridinoline</td>
</tr>
<tr>
<td>RER</td>
<td>rough endoplasmatic reticulum</td>
</tr>
<tr>
<td>RF</td>
<td>rheumatoid factor</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SF</td>
<td>synovial fluid</td>
</tr>
<tr>
<td>SP4</td>
<td>synthetic peptide 4; a linear peptide with amino acid sequence SAGFDFSLPQPPQEKY</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitor of matrix metalloproteinase</td>
</tr>
<tr>
<td>TPK</td>
<td>N-tosyl-L-phenylalanine chloromethyl ketone</td>
</tr>
<tr>
<td>uPA</td>
<td>urokinase-type plasminogen activator</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
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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. 1983).
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 \( \alpha \)-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 \( \alpha \)-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 \( \text{Fe}^{2+} \), \( \alpha \)-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 enzyme 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 \( \alpha \)-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 \( \beta \)-
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 enzyme 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 NaBH₄. 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.
2.5.3 The mature, trivalent cross-links

The mature cross-linking residues on the hydroxylysine aldehyde pathway are trivalent 3-hydroxy-3-pyrrolidin-2-yl 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 histidinohydroxylysiononorleucine (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, 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 Delaissé 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-P₁-P₂-P₃-AMC show that an amino acid with hydrophilic side chain such as Arg or Lys in P₁ along with an amino acid having a small, hydrophobic side chain within P₂ 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 P₂ 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). Glycine is accepted in the P₁ 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) cannot 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.

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

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 PRGVPD 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, although 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
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, bisphosphonates 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 EKAHβDGGGR 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 Arg22 and Tyr23 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 divalentely 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 Gynecology, 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 NaBH₄ (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 NaBH₄. 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 collagenous 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 collagenous 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® C₁₈ 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, C₅ 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 divalent 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 NaBH₄ 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: SAGDFDFSFLPPQEQY.

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 Na₂HPO₄ 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 ¹²⁵I by the chloramine-T method. The labelled antigen was separated from free iodine using a SEP PAK C₁₈ 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 (SAGDFDFSFLPQPPQEKY; produced by Neoysystem Laboratories, Strasbourg, France), derived from the carboxyterminal telopeptide region of type I collagen as a tracer and standard antigen. The antiserum (²#239) used was produced in a rabbit against the divalent 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® 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 Calcanyl 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 CaCl₂ (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 (NH₄)₂CO₃, 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 C₈ 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

\[
\text{T(GXY)$_7$SAGFDFSLPQPPQEKAH\beta DGGR}
\]

GLPGLTAGLPGMKHR (or \(\alpha_2\)-chain)

Trivalently cross-linked ICTP from bovine bone

\[
\text{T(GXY)$_7$SGGYDLSFLPQPPQEKAH\beta DGGR}
\]

GLPGLTAGLPGMKHR (or \(\alpha_2\)-chain)

HHL-cross-linked ICTP from human skin

\[
\text{T(GXY)$_7$SAGFDFSLPQPPQEKAH\beta DGGR}
\]

\[
\text{GHNGLDGLK}
\]

GLPGLTAGLPGMKHR

Synthetic peptide SP4

\[
\text{SAGFDGSGLPQPPQETY}
\]

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 divalentely 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 $\alpha_1C\alpha_1C\alpha_1H$ and 10421 for the form $\alpha_1C\alpha_1C\alpha_2H$. 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 (dehydrolysinoonorleucine LNL, dehydrohydroxylysinoonorleucine HLNL, dehydrodihydroxylysinoonorleucine 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 carboxyterminal 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.

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

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

<table>
<thead>
<tr>
<th>Physiological turnover (cathepsin K)</th>
<th>Pathological degradation (MMPs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PINP</td>
<td>PIIINP</td>
</tr>
<tr>
<td>CrossLaps</td>
<td>ICTP</td>
</tr>
<tr>
<td>NTx</td>
<td>PYD</td>
</tr>
<tr>
<td>Total Dpd</td>
<td>Free Dpd</td>
</tr>
</tbody>
</table>

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 probably measures the MMP-derived degradation products.
References


