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CARBOXYTERMINAL
TELOPEPTIDE STRUCTURES
OF TYPE I COLLAGEN IN
VARIOUS HUMAN TISSUES

FACULTY OF MEDICINE,
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STRUCTURES OF TYPE I COLLAGEN
IN VARIOUS HUMAN TISSUES**

Academic dissertation to be presented with the assent of the Faculty of Medicine of the University of Oulu for public defence in the Great Hall of the Utsjoki Parish Hall (Pedar Jalven tie 1, Utsjoki), on 20 August 2010, at 12 noon

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Abstract

Type I collagen is the main connective tissue protein in vertebrates. The cross-linking and correct organisation of the molecules is crucial for the proper function of the tissue. Traditionally collagen cross-linking has been studied using chemical cross-link analyses. However, this does not distinguish between the collagen types or the location of the cross-link within the molecule. The focus in this work was to study the carboxyterminal telopeptide domain of type I collagen for the differently cross-linked forms. An immunochemical approach was used and a new immunoassay, SP4, was developed for the detection of immaturely cross-linked peptide forms. The differently cross-linked structures were purified and characterised from human bone by using SP4 together with the earlier developed ICTP assay for trivalently cross-linked C-terminal telopeptide form. It was found that the majority of the trivalent cross-links in the C-terminal telopeptide were presently unknown structures, other than pyridinoline. A non-cross-linked form of C-terminal telopeptide of $\alpha 1$ -chain of type I collagen was also discovered in bone. The epitope of the ICTP assay was characterised and found to reside in the phenylalanine rich region of the ICTP peptide. MMP-9, but not cathepsin K, mediated breakdown of the collagenous matrix was found to produce a peptide detectable by the ICTP assay.

Healthy human Achilles tendon comprises mainly of type I collagen. In ruptured Achilles tendons, an increased type III collagen content was found. Since the synthesis of type III collagen was not increased, it is postulated that the type III collagen must have accumulated over a long period of time indicative of a long-lasting microtraumatic process in the tendon before the total rupture occurred.

The ICTP content was increased and the ratio of SP4 to ICTP decreased in calcified stenotic aortic valves suggesting a change in the molecular organisation and cross-linking towards the type found in human bone. The total collagen content was dramatically decreased in the calcified valves.

Both in the Achilles tendons and in the aortic valves, the ICTP content was found to decrease with age with a concomitant increase in the variants of the C-terminal telopeptide structures detectable with the SP4 assay, pointing to a change in the molecular organisation of the collagenous matrix in these tissues.

Keywords: Achilles tendon rupture, aortic valve stenosis, bone, calcification, collagen, cross-linking

To my beloved family

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Utsjoki May 2010

Heidi Eriksen

Abbreviations

| | |
|-------------|---|
| ACP | aldol condensation product |
| deH-DHLNL | dehydro–dihydroxylysinoonorleucine |
| deH-HLNL | dehydro–hydroxylysinoonorleucine |
| DHLNL | dihydroxylysinoonorleucine |
| ECM | extracellular matrix |
| EDTA | ethylenediamine tetra-acetic acid |
| HHL | histidinohydroxylysinoonorleucine |
| HHMD | histidinohydroxymerodesmosine |
| His | histidine |
| HLNL | hydroxylysinoonorleucine |
| HL-Pyl | hydroxylysyl pyrrole |
| HP | hydroxylysyl pyridinoline |
| HPLC | high performance liquid chromatography |
| Hyl | hydroxylysine |
| ICTP | cross-linked carboxyterminal telopeptide of type I collagen |
| IIINTP | aminoterminal telopeptide of type III collagen |
| LH | lysyl hydroxylase |
| LOX | lysyl oxidase |
| LOXL | lysyl oxidase like protein |
| LP | lysyl pyridinoline |
| L-Pyl | lysyl pyrrole |
| Lys | Lysine |
| pC-collagen | collagen molecule with retained carboxyterminal propeptide |
| PICP | carboxyterminal propeptide of type I collagen |
| PIIINP | aminoterminal propeptide of type III collagen |
| PINP | aminoterminal propeptide of type I collagen |
| PLOD | lysyl hydroxylase gene |
| pN-collagen | collagen molecule with retained aminoterminal propeptide |
| SP 4 | synthetic peptide 4 |
| PBS | phosphate buffered saline |

List of original publications

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

- I Eriksen HA, Sharp CA, Robins SP, Sassi ML, Risteli L & Risteli J (2004) Differently cross-linked and uncross-linked carboxyterminal telopeptides of type I collagen in human mineralized bone. *Bone* 34: 720–727.
- II Sassi ML, Eriksen H, Risteli L, Niemi S, Mansell J, Gowen M & Risteli J (2000) Immunochemical characterization of assay for carboxyterminal telopeptide of human type I collagen: Loss of antigenicity by treatment with cathepsin K. *Bone* 26: 367–373.
- III Eriksen HA, Pajala A, Leppilahti J & Risteli J (2002) Increased content of type III collagen at the rupture site of human Achilles tendon. *J Orthop Res* 20: 1352–1357.
- IV Eriksen HA, Satta J, Risteli J, Veijola M, Väre P & Soini Y (2006) Type I and type III collagen synthesis and composition in the valve matrix in aortic valve stenosis. *Atherosclerosis* 189(1): 91–98.

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1 Introduction

The elaborate extracellular matrix (ECM) of connective tissues consists of insoluble fibers, microfibrils, a wide range of soluble proteins and glycoproteins. Its function is not only to provide the tissues with their mechanical and physicochemical properties but also to provide a suitable environment for normal cellular function. ECM controls many aspects of cell behaviour such as attachment, migration, differentiation and gene expression.

Type I collagen is the main constituent of the ECM in most tissues in vertebrates. In the skeletal tissues like bone, tendons, and ligaments, it is the main collagen type present and responsible for the tensile strength of the tissue. The type I collagen molecule is formed when the propeptide domains at both ends of the molecule are removed. After secretion outside the cell, the collagen molecules are enzymatically cross-linked to each other in the fibrils. The cross-links are formed between certain lysine and hydroxylysine residues within the helical and telopeptide domains of the collagen molecule. Helical histidine residue can also be involved under certain circumstances. The cross-links provide the collagen matrix with resistance against proteolytic enzymes and increase the mechanical properties of the tissue. The collagen cross-linking differs in tissues depending on their functional and mechanical demands. Two main cross-linking pathways exist – one based on lysine aldehydes and the other on hydroxylysine aldehydes. The mature trivalent cross-links of these routes are histidinohydroxylysinonorleucine (HHL) and pyridinoline and pyrrole structures.

In the present study, differently cross-linked C-terminal telopeptides have been purified and characterised using immunoassays specific for the trivalently cross-linked structure, ICTP, and by developing a new immunoassay SP4 for the detection of the immaturely cross-linked or even non-cross-linked forms of C-telopeptide of type I collagen. These assays have been used to study the collagenous matrix of healthy human bone. The changes in the collagenous matrix were also examined in pathological situations such as ruptured Achilles tendons and stenotic aortic valves with extensive calcification.

2 Review of the literature

2.1 The collagen protein family

Collagens are present in all vertebrate phyla (van der Rest & Garrone 1991) and have also been identified in lower organisms such as fungi (Celerin *et al.* 1996) suggesting that collagens may have evolved from a common ancestor prior to the divergence of animals and fungi. Collagens are the major structural connective tissue components, constituting almost one third of the total protein mass in vertebrates (Robins 1988). The primary structure of collagens is characterised by repeated triplets of the Gly-X-Y sequence, which form the α -chains. The α -chains form a left handed triple helix in which every third residue comes into the centre and this results in formation of a right-handed superhelix. Because of steric reasons, the centre of the helix can be occupied only by glycine residues. The α -chains contain high amounts of imino acids, with the X position often being occupied by proline (about 120 residues per α -chain) and the Y position by 4-hydroxyproline (about 100 residues per α -chain). The side chains of amino acids at the X and Y positions in the triple helix point outward from the helix and offer the triple helix exceptional potential for undergoing lateral electrostatic interactions, particularly with other triple helices. All of the α -chains also contain non-helical telopeptide regions, which differ extensively between different collagen types. (Brown & Timpl 1995)

So far 28 distinct genetic collagen types consisting of at least 46 distinct polypeptide chains have been characterised in vertebrates (Shoulders & Raines 2009). In addition, there are several proteins that have collagen-like domains, including complement component C1q (Kaul & Loos 1995), the tail structure of acetylcholine esterase (Krejci *et al.* 1997), the collectins (mannose-binding protein, lung surfactant proteins SP-A and SP-D, collectin-43, conglutinin), the ficolins (Lu *et al.* 2002), type I and II scavenger receptor of macrophages (Freeman *et al.* 1990), receptor MARCO present in macrophages (Elomaa *et al.* 1995), adiponectin (Okamoto *et al.* 2000), a src-homologous-and-collagen protein (Pelicci *et al.* 1992), aggretin (Chung *et al.* 1999) and ectodysplasin (Ezer *et al.* 1999). However, these are not classified as collagens as they are not structural components of the ECM.

The different collagen types in mammals can be classified into subgroups according to their structure, supramolecular organisation and function. Collagen

types I, II, III, V and XI make up the group of fibril forming collagens, the rest being classified as non-fibril forming collagens. The latter group can be further divided into subgroups of FACIT (fibril-associated collagens with interrupted triple-helices) (types IX, XII, XIV, XVI and XIX), collagens forming hexagonal networks (types VIII and X), collagens found in basement membranes (type IV), collagen forming beaded filaments (type VI), collagen forming anchoring fibrils for basement membranes (type VII), collagens with transmembrane domains (types XIII and XVII) and the MULTIPLEXINs (proteins with multiple triple-helix domains and interruptions) (types XV and XVIII).

For reviews, see Kielty *et al.* 1993, Prockop & Kivirikko 1995, Bateman *et al.* 1996, Myllyharju & Kivirikko 2001, Shoulders & Raines 2009.

2.2 Fibrillar collagens

Quantitatively the fibrillar collagens type I, II, and III account for more than 70% of the total collagens in the body (Kühn 1987). The molecules are composed of a long triple helical domain of about 1000 amino acids with short (about 20 amino acids) non-helical telopeptide domains at either end. The length of the collagen molecule is about 300 nm, and its diameter about 1.4 nm. The molecules are synthesised as procollagens with large propeptides at both ends. After secretion from the cell into the extracellular space, the N- and C-terminal propeptides are removed by specific proteases to generate the collagen monomers, which then assemble to form fibrils. The telopeptides at both ends of the collagen monomers vary in their primary structure between different α -chains and collagen types. Their main function in type I, II and III collagens is to stabilise the newly synthesised collagen fibrils by intermolecular covalent cross-links (Eyre 1987).

Type I collagen is the principal fibrillar component in many tissues, especially in tissues prone to high mechanical forces such as bone, tendon and ligament. Together with type III collagen, it is also the major collagen type in many elastic tissues like skin, blood vessels and heart valves. The classical heterotrimeric type I collagen is composed of two α 1(I)-chains and one α 2(I)-chain encoded by COL1A1 and COL1A2 genes. Minor amounts of homotrimeric type I collagen composed of three α 1(I)-chains are found in embryonic tissues (Pucci-Minafra *et al.* 1993), in some malignancies (Pucci-Minafra *et al.* 1998, Kauppila *et al.* 2001), and this form is suspected to be present in osteoporotic (Mann *et al.* 2001) and osteoarthrotic (Bailey *et al.* 2002) bone matrixes. Type I collagen is often associated with smaller quantities of type III and type V

collagens, so that individual fibrils can consist of all three types. Such heterotypic interactions may regulate the diameter of a fibril. (Birk *et al.* 1990) Mutations in the genes coding for type I collagen are responsible for the different forms of osteogenesis imperfecta (OI) and for some forms of Ehlers-Danlos syndrome (EDS) (Byers 2000).

Type III collagen is mainly found in soft connective tissues, such as blood vessels and granuloma tissue and gives the tissue elastic properties and it is also important in wound healing. Type III collagen is a homotrimer of three identical $\alpha 1(\text{III})$ -chains encoded by COL3A1 gene. Type II collagen is formed by three $\alpha 1(\text{II})$ -chains and is the major constituent of cartilage, vitreous body, cornea and sclera. In cartilage it forms the network in which large proteoglycans exist, which is essential for the resilience of cartilage. Type V occurs in association with type I and type III collagen. Type XI collagen is found in cartilage in association with type II collagen. (Brown & Timpl 1995)

2.3 Biosynthesis and assembly of type I collagen

Collagen biosynthesis is a complex process involving several translational and posttranslational modifications. After transcription, processing and transportation of the mRNA to the cytoplasm, the formation of prepro α -chains begins with the ribosomal synthesis of the short hydrophobic N-terminal signal peptide which interacts with the signal recognition particle. The complex is bound to the endoplasmic reticulum (ER), where the translation continues, directing the forming α -chain into the lumen of ER, where the signal peptidase cleaves off the signal peptide.

The intracellular post-translational modifications to the α -chains take place in the lumen of the ER and begin during translation. Some proline residues at the Y-position of the Gly-X-Y repeats are converted to 4-hydroxyproline and at the X-position to 3-hydroxyproline, by the enzymes prolyl-4-hydroxylase and prolyl-3-hydroxylase, respectively. Appropriate levels of 4-hydroxyproline are crucial for regulating the thermal stability of the collagen triple helices. Certain lysine residues in the helix and the non-helical telopeptide domains are hydroxylated by the different isoenzymes of lysyl hydroxylase. An enzyme, hydroxylysyl galactosyltransferase, can add galactosyl groups to the hydroxylysine residues. The formed galactosylhydroxylysine can be further converted to a disaccharide glucosylgalactosyl hydroxylysine by galactosyl hydroxylysyl glucosyltransferase. The function of glycosylation is unknown, but it has been speculated to control

the collagen fibril diameter (Torre-Blanco *et al.* 1992). High-mannose type oligosaccharides are attached to certain asparagine residues in the C-propeptides by the oligosaccharyl transferase complex. The high-mannose oligosaccharide has been postulated to have a role in the clearance of the C-propeptide from the circulation (Smedsrød *et al.* 1990, Lee *et al.* 2002). The enzyme protein disulfide isomerase catalyses the formation of disulfide bonds within and between the formed α -chains. The *cis-trans* isomerisation of the prolyl residues is catalysed by *cis-trans*-isomerase. This may regulate the optimal folding of the collagen molecules, as the formation of triple helix requires that any *cis*-peptide bonds are converted into the *trans*-form (Bächinger *et al.* 1978). In the lumen of ER, molecular chaperones such as HSP47 and polypeptide chain binding protein, associate with the procollagen chains to assist and regulate the assembly of the individual α -chains in the triple helical molecules. The assembly of the α -chains begins by folding of the C-propeptides, which then steer the triple helix formation towards the N-terminal end in a zipper-like fashion. Interchain disulfide bonds are formed to stabilise the folded structure.

The folded procollagen molecules are transported through the Golgi complex, where they are packaged into secretory vacuoles and released into the extracellular space by exocytosis.

See Brown & Timpl 1995, Bateman *et al.* 1996 for reviews.

2.4 Fibril formation

Fibril formation starts extracellularly with the cleavage of the propeptides by specific endopeptidases, the procollagen N-proteinase and the procollagen C-proteinase. The cleavage of the globular carboxyterminal propeptide is essential for the occurrence of fibril formation, as pC-collagen is not capable of being incorporated into fibrillar collagen matrix (Kadler *et al.* 1987). The aminoterminal propeptide cleavage is not as crucial but it has a role in the regulation in fibril morphology.

In the extracellular space, the collagen molecules self-assemble into specifically staggered configurations via hydrophobic and electrostatic interactions between the adjacent chains (Yamauchi & Mechanic 1988). The α 2-chain of type I collagen, being more hydrophobic than the α 1-chain, plays an important role in the self-assembly of collagen molecules into fibrils, as it has been shown that the efficiency of the assembly is markedly reduced in homotrimeric type I collagen (McBride *et al.* 1992, Han *et al.* 2008). Although

the exact mechanism remains obscure, the telopeptide domains seem to have a catalytic role in the self-assembly process, as the removal of the telopeptides by pepsin digestion changes the kinetics of fibrillogenesis, but does not interfere with the assembly of the molecules into fibers. The triple helical domains seem to carry the information for noncovalent interactions and correct fiber assembly. (Kuznetsova & Leikin 1999) Collagen molecules in the fibers are axially organised in an end-overlap manner, the molecules in the fibril being staggered about one quarter. This D-spacing (1 D = about 65–67 nm, collagen molecule is 4.4 D long) has been reported to vary from tissue to tissue, bone, tendon, cartilage and dura mater being reported to have a d-periodicity of 67 nm. The assembled collagen molecules within the fibrils are covalently cross-linked to each other (discussed in more detail in section 2.5.).

Individual fibrils can consist of several different fibrillar collagen types, e.g. type I and III collagens in human skin (Fleischmajer *et al.* 1990). Other extracellular matrix components, such as non-fibrillar collagens and some glycoproteins and proteoglycans that are able to associate with fibrillar collagens contribute to the fibril formation and result in the divergence in fibril organisation between tissues. The ECM, instead of being simply a scaffold, provides a number of signals to cells, that regulate all aspects of their phenotype from morphology to differentiation. This is committed through the action of so called matricellular proteins, that are a class of ECM related molecules defined through their ability to modulate cell-matrix interactions (for further reading see Bornstein *et al.* 2004, Alford & Hankenson 2006).

For reviews, see Bateman *et al.* 1996, Lamandé & Bateman 1999, Robins 1999.

2.5 Collagen cross-linking

Collagen cross-linking can occur through two different mechanisms: enzymatic and non-enzymatic. Enzymatic cross-linking takes place during development and maturation. It is essential for the stabilisation of the collagenous matrix and is responsible for the tensile strength of the tissues (for reviews see Robins & Brady 2002, Eyre 1987, Yamauchi & Mechanic 1988, Bailey *et al.* 1998, Robins 2007). In non-enzymatic cross-linking, the sugar residues (glucose, ketose or other metabolic intermediate) condense with the collagen molecule resulting in the formation of advanced glycation end products (AGEs). This often occurs between collagen helices and does not involve the telopeptides. The formation of AGEs

occurs during ageing and is one of the causes of dysfunction of collagenous tissues in old age (for further reading, see Paul & Bailey 1996, Bailey *et al.* 1998, Saito & Marumo 2010).

2.5.1 Enzymatic cross-linking

Enzymatic cross-linking plays a crucial role in determining the characteristic physical and chemical properties of collagen. The cross-links formed are related to the physiological functions of the tissues and not to the particular genetic type of collagen. Two enzymes play critical roles in the mechanism of enzymatic cross-linking. Lysyl hydroxylase determines the types of cross-links formed by specifying the cross-link formation pathway of the tissue whilst lysyl oxidase initiates the cross-link formation reactions by converting certain lysine residues to reactive aldehyde forms.

In the quarter-staggered end-overlap arrays of fibrillar collagen molecules, the cross-links form in specific positions. In the α -chains of type I collagen, there are lysine or hydroxylysine residues at five different locations that participate in enzymatic cross-linking; three in the telopeptides (residue 9 in the α 1-chain and 5 in α 2-chain in the N-terminal telopeptide and residue 16 in the α 1-chain in the C-terminal telopeptide) and two helical residues at positions 87 and 930. The helical histidine residue at position 92 is also involved in cross-linking mediated by the lysine aldehyde pathway (Yamauchi *et al.* 1987). At the C-terminal telopeptide, only the α 1-chains are involved in cross-linking, since the α 2-chain lacks a lysine residue. Despite the fact that it is not directly involved in cross-linking, the C-terminal telopeptide of α 2-chain may still have a role in cross-link formation. Since the α 2(I)-C-telopeptide is shorter than the C-telopeptides of α 1-chains, it has been proposed as being necessary in accommodating the folded C-telopeptides of α 1-chains. The folding of the α 1-chains is thought to arrange the lysines in an appropriate register with the helical region of adjacent molecules to facilitate cross-linking. (Orgel *et al.* 2000)

2.5.2 Lysyl hydroxylase

The hydroxylation level of the telopeptide-located lysine residues in the collagen molecule determines the cross-linking pathway that will be followed (Robins & Brady 2002) (figure 1). Lysyl hydroxylase (LH) (procollagen-lysine, 2-oxoglutarate 5-dioxygenase, EC 1.14.11.4) is an enzyme that catalyses the

hydroxylation of lysine residues in collagen molecules. Three isoenzymes LH1, LH2 and LH3, originating from different genes PLOD1-3, have been characterized to date (Hautala *et al.* 1992, Valtavaara *et al.* 1997, Valtavaara *et al.* 1998). In addition, LH2 occurs in two alternatively spliced forms, LH2a and LH2b, of which the LH2b is more common in adult human tissues (Yeowell & Walker 1999). The different isoenzymes possess tissue and cell-specific patterns of expression in adult mouse (Salo *et al.* 2006). No collagen type specificity has been found in the action of the LH (Wang *et al.* 2000), although a clear preference for some sequences to be bound and hydroxylated by a certain isoenzyme has been proposed (Risteli *et al.* 2004).

As long as a few decades ago, it was speculated that LH isoenzymes specific for helical and telopeptide regions of the collagen molecule exist, as the amino acid sequences differ in these regions (Royce & Barnes 1985). During recent years, it has been shown that the LH2 isoenzyme hydroxylates the telopeptide-located lysine residues. Fully differentiated human osteoprogenitor cells (bone marrow stromal cells), which are able to undergo osteoblastic differentiation and bone formation, have been reported to express LH gene PLOD2, whereas normal skin fibroblasts did not. This coincided with the onset of matrix mineralization and with the hydroxylation of telopeptidyl lysines (Uzawa *et al.* 1999). In Bruck-syndrome, where collagen cross-linking is defective in bone, but not in cartilage or tendon, mutations in PLOD2 have been characterised indicating that tissue specificities occur in the LH2 expression. Bruck-syndrome is characterized by osteoporosis, long bone bowing and scoliosis due to vertebral deformities and congenital joint contractures. (Bank *et al.* 1999a) Increased expression of PLOD2 was found also in fibrotic skin of patients with systemic sclerosis, supporting the hypothesis that LH2 is the telopeptidyl LH, which determines the cross-linking pathway (van der Slot *et al.* 2003). Recent evidence has indicated that specifically the LH2b may direct the collagen cross-linking pathways through its action on telopeptidyl lysine residues (Pornprasertsuk *et al.* 2004). However, its level of activity seems to be critical in proper fibril formation and accumulation of mineral, since overexpression of LH2b has shown to lead to defective collagen fibrillogenesis and matrix mineralisation of MC3T3-E2-derived osteoblastic cells (Pornprasertsuk *et al.* 2005).

Mutations in the gene coding for LH1 (PLOD1) have been found in Ehlers-Danlos syndrome type VI, where defective lysine hydroxylation occurs within the helical regions, but is normal in the telopeptide domains (Yeowell & Walker 2000).

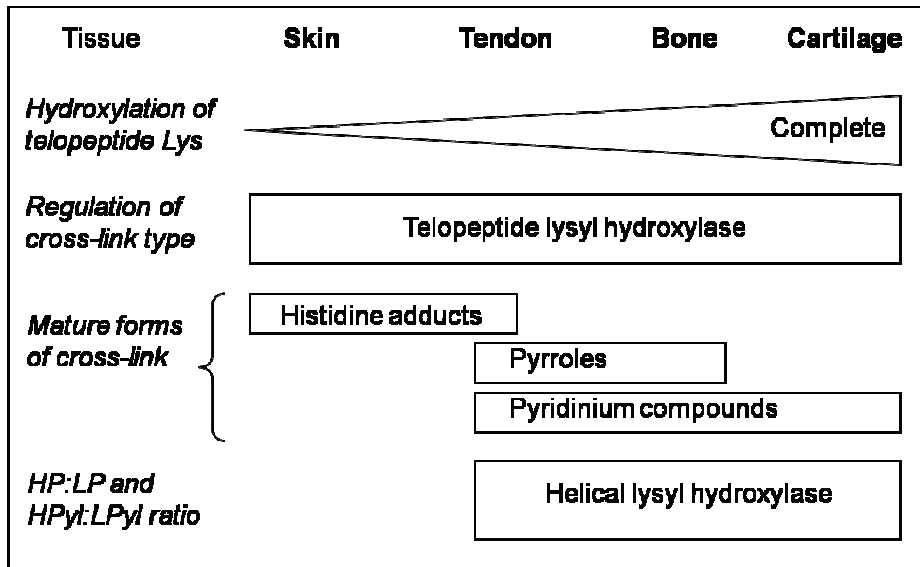


Fig. 1. The spectrum of effects of lysine hydroxylation in cross-linking in different tissues. (Robins & Brady 2002, published by kind permission of Elsevier).

The LH3 isoenzyme has been found to possess, in addition to LH activity, also hydroxylysyl galactosyltransferase (Wang *et al.* 2002) and galactosylhydroxylysyl glucosyltransferase activities (Heikkinen *et al.* 2000). As such, LH3 is able to perform all the steps needed for the addition of carbohydrate residues to fibrillar collagens. In fact, LH3 seems to be the main source for galactosylhydroxylysyl glucosyltransferase activity in mouse tissues. This ability of LH3 is essential for normal development and its lack is lethal in mouse embryos due to defective basement membranes. (Ruotsalainen *et al.* 2006)

Increased hydroxylation levels of telopeptide but not helix located lysines have been reported in the callus of healing bone fracture (Wassen *et al.* 2000), transiently in wound healing (Bailey *et al.* 1975) and chicken tendon hypertrophy (Gerriets *et al.* 1993). Increased lysyl hydroxylation levels in the entire collagen molecule have been reported in fibrotic skin from a patient with lipodermatosclerosis (Brinckmann *et al.* 1999). Increased levels of helical lysyl hydroxylation in collagen have been reported in keloid, thick scar tissues of human skin (Uzawa *et al.* 1998) and degenerative supraspinatus tendons (Bank *et al.* 1999). An interesting study of the possible mechanism of spaceflight induced osteopenia was published by Saito *et al.* (2003), who reported that hypergravity

upregulated telopeptidyl LH expression, whereas microgravity stimulated helical lysyl hydroxylation in MC3T3-E1 cell cultures, thus altering the post-translational modifications of the collagen involved in cross-link formation.

2.5.3 Lysyl oxidase

Enzymatic cross-link formation in collagen and elastin is initiated extracellularly by the enzyme lysyl oxidase (LOX) (protein lysine 6-oxidase, EC 1.4.3.13). The substrate of LOX is the collagen molecule in the early stages of $\frac{1}{4}$ D-staggered fibril formation. LOX oxidatively deaminates the ϵ -amino groups in the lysine or hydroxylysine residues present in the telopeptide domains of collagen converting them to the corresponding aldehydes, allysine (Lys^{ald}) and hydroxyallysine (Hyl^{ald}). After the aldehydes are formed, di-, tri- and tetravalent cross-links form spontaneously as a consequence of specific alignment of the collagen molecules in the fibrils. Copper is prerequisite for the function of lysyl oxidase. Five different isoenzymes have been characterised, namely lysyl oxidase (LOX), and lysyl oxidase like proteins 1 to 4 (LOXL1-4). See Csiszar 2001, for review.

The procollagen C-proteinase processes prolysyl oxidase into its active form in the ECM (Uzel *et al.* 2001). Thus, in addition to the LOX mRNA expression rate, the activity of procollagen C-proteinase may be an important determinant of active LOX in tissues (Uzel *et al.* 2000). Increased activity of LOX is related to fibrotic conditions (Desmouliere *et al.* 1997, Peyrol *et al.* 1997). Reduced activities of LOX have been reported in impaired copper metabolism (Menkes' disease and cutis laxa), lathyrism and in malignantly transformed cells (see Smith-Mungo & Kagan 1998). There is evidence to associate LOX with other biological functions in addition to collagen and elastin cross-linking, such as activation the transcription of type III collagen gene promoter (Giampuzzi *et al.* 2000), cell growth and differentiation (Csiszar 2001), intranuclear functions (Li *et al.* 1997) and suppression of the ras oncogene (Smith-Mungo & Kagan 1998).

2.5.4 The cross-linking pathways

The fibrillar collagens are enzymatically cross-linked by two different pathways based either on lysine aldehyde (Lys^{ald}) or hydroxylysine aldehyde (Hyl^{ald}) derived cross-linking (figure 2). The Hyl^{ald} pathway predominates in tissues such as cartilage, bone, dentin, most tendons, ligament and embryonic skin. Lys^{ald} - based cross-linking prevails in soft connective tissues such as skin, cornea, rat tail

tendon and sclera. Skin, with virtually no hydroxylation of the telopeptide located lysines and cartilage with complete hydroxylation level represent the extremes of these cross-linking pathways (figure 1). However, in many tissues both cross-linking pathways operate to some extent in parallel. The cross-links formed in these pathways differ in the chemical structure and also by the location of the cross-link in the molecule. There is an association between collagen cross-linking type with certain kind of organisation of the collagen molecules within the fibres. (Yamauchi & Mechanic 1988, Wassen *et al.* 2000, Knott *et al.* 1997)

2.5.5 Divalent cross-links

In the Hyl^{ald} pathway, the telopeptide-located Hyl aldehyde is combined with a Hyl or Lys residue located in the helix. This leads to formation of a Schiff's base which rapidly undergoes Amadori rearrangement to form the keto-imine structures hydroxylysino-5-ketonorleucine (HLKNL) and lysino-5-ketonorleucine (LKNL), respectively. *In vitro*, the reduction with NaBH₄ stabilises these structures forming the acid and heat stable derivatives dihydroxylysino-5-ketonorleucine (DHLNL) and hydroxylysino-5-ketonorleucine (HLNL).

When a telopeptide located Lys^{ald} reacts with a helical Hyl or Lys residue, this results in the formation of divalent aldimine cross-link dehydro-hydroxylysino-5-ketonorleucine (deH-HLNL) and dehydro-lysino-5-ketonorleucine (deH-LNL). As these moieties lack the hydroxyl group, they do not undergo the Amadori rearrangement and remain in the unstable Schiff's base form. Upon *in vitro* reduction with borohydride, these form hydroxylysino-5-ketonorleucine (HLNL) and lysino-5-ketonorleucine (LNL), respectively. Two lysine aldehydes at the N-terminal telopeptide have been suggested to react and form the intramolecular aldol-condensation product (ACP).

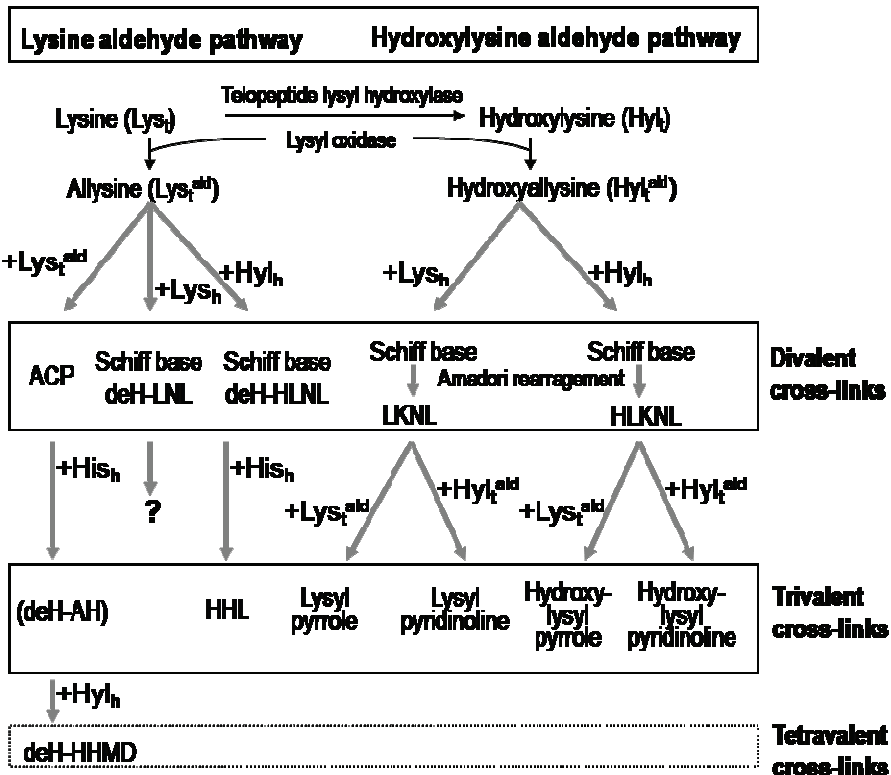


Fig. 2. Collagen cross-linking pathways. Abbreviations: Lys = lysine, Hyl = hydroxy-lysine, ald = aldehyde, ACP= aldol condensation product, deH-LNL = dehydroxy-lysinonorleucine, deH-HLNL = dehydroxy-hydroxylysinonorleucine, LKNL = lysine-keto-norleucine, HLKNL = hydroxylyxino-keto-norleucine, deH-AH = dehydroaldohistidine, HHL = histidino-hydroxylysinonorleucine, deH-HHMD = dehydrohistidinohydroxymerodesmosine. The subscript t or h indicates the telopeptide or helical location of the corresponding residue.

The reduced form of the keto-imine cross-link, HLNL is a structural isomer of the corresponding aldimine form, and they co-elute in cross-link analyses (Robins & Bailey 1975, Knott & Bailey 1998). The relative proportions of HLNL derived from the aldimine or keto-imine forms have been studied using periodate degradation. In bovine Achilles tendon, the aldimine form predominated (90%), whereas in bone, the keto-imine form was more common (70%) of the HLNL cross-link. (Robins & Bailey 1975)

Reduced amounts of divalent cross-links in bone reduce its mechanical properties (Oxlund *et al.* 1995, Oxlund *et al.* 1996). Their increased amounts in tissues also reflect an increased turnover rate, as has been shown with osteoporotic bone (Mansell & Bailey 2003). The presence of divalent cross-links has been reported to increase the resistance towards collagenolytic enzymes (Vater *et al.* 1979).

Table 1. The reported contents of different collagen cross-links in various human tissues, presented as moles per mole of collagen.

| Tissue | Divalent | | Trivalent | | | Ref |
|--|----------|------|-----------|-------|----------|-----|
| | DHLNL | HLNL | HP | LP | Pyrroles | |
| Bone | | | | | | |
| Femoral heads* | | | | | | |
| Normal | 0.22 | 0.14 | 0.260 | 0.090 | present | 1 |
| Osteoarthrotic | 0.37 | 0.13 | 0.250 | 0.070 | present | 1 |
| Osteoporotic | 0.12 | 0.18 | 0.270 | 0.120 | | 6 |
| Vertebrae* | | | | | | |
| Normal | | | 0.120 | 0.060 | present | 5 |
| Osteoporotic | | | 0.090 | 0.040 | present | 5 |
| Bone (n=10, age 48–90 years) | | | 0.197 | 0.055 | | 4 |
| Cartilage | | | | | | |
| Articular (neonate) | 2.4 | 0 | 0.6 | 0 | | 2 |
| Articular (16 years) | 0.2 | 0 | 2.30 | 0 | | 2 |
| Tendon (n=10, 48–90 years) | | | 0.547 | 0.019 | | 4 |
| Aorta (non calcified, mean age 71 years) | | | 0.460 | 0.090 | | 3 |
| Aorta (calcified lesion) | | | 0.570 | 0.040 | | 3 |
| Aorta (n=10, age 48–90 years) | | | 0.201 | 0.046 | | 4 |
| Kidney (n=10, age 48–80 years) | | | 0.302 | 0.042 | | 4 |
| Liver (n=10, age 48–90 years) | | | 0.405 | 0.044 | | 4 |

*contains both the mineral protected and non-mineralised collagen fractions of the bone tissue.

References 1. Mansell *et al* 1998, 2. Eyre 1987, 3. Hoshino *et al* 1995, 4. Gineyts *et al* 2000, 5. Oxlund *et al* 1996, 6. Mansell & Bailey 2003.

2.5.6 Trivalent cross-links

The pyridinium-derived trivalent cross-links hydroxylysylpyridinoline (HP) (Fujimoto *et al.* 1978) and lysylpyridinoline (LP) (Ogawa *et al.* 1982) are maturational products of the Hyl^{ald} pathway. These forms originate from the condensation of the keto-imine cross-link with telopeptide-located Hyl^{ald}. Some authors have suggested that the additional telopeptide-located Hyl^{ald} derives from

another keto-imine cross-link, which would result in a cross-link between three collagen molecules (Eyre *et al.* 1988). However, it has also been proposed that there is the involvement of free Hyl^{ald}, which would allow the pyridinoline to connect two molecules (Robins & Duncan 1983). The pyridinolines are naturally fluorescent compounds. There is also some evidence for a non-fluorescent deoxyanalogue of pyridinolines present in skin (Tilson *et al.* 1985). The activity of lysyl hydroxylase responsible for the helical lysine hydroxylation, determines the ratio of HP to LP (Eyre *et al.* 2002, Robins & Brady 2002). HP is widely present in many tissues, whereas bone and dentin feature with relatively low helical lysyl hydroxylation level and thus the LP form is more abundant in these tissues, their ratio in human bone being about 3-4:1 (Eyre *et al.* 1988) (table 1). In bone, the N-terminal telopeptide contains relatively more LP than the C-terminal telopeptide (about two thirds of total LP), although the total pyridinoline type cross-links appear equally in N- and C-terminal telopeptide domains (Hanson & Eyre 1996).

Another interesting feature of bone is the stoichiometric deficiency of trivalent pyridinoline cross-links (table 1) (Robins & Duncan 1987, Eyre *et al.* 1988), which has led to the discovery and characterisation of another mature cross-link in bone having a pyrrole structure (Scott *et al.* 1983, Kuypers *et al.* 1992, Hanson & Eyre 1996, Brady & Robins 2001). The pyrrole type cross-link also exists as differently hydroxylated variants, hydroxy-lysylpyrrole and lysylpyrrole. They are labile to acid and alkali hydrolysis which has complicated their isolation and characterisation (Robins & Brady 2001). The pyrrole type cross-links have been proposed to originate from a keto-imine cross-link condensing with a telopeptide located Lys^{ald} (Kuypers *et al.* 1992, Hanson & Eyre 1996), thus requiring both Hyl^{ald} and Lys^{ald} derived products (Brady & Robins 2001). Pyrrole cross-links have been identified in mineralising tissues (Hanson & Eyre 1996) and in tendon (Kuypers *et al.* 1992), but not in skin or cartilage (Knott & Bailey 1998). About 85% of the total pyrrole cross-links are present in the N-terminal telopeptide domain (Hanson & Eyre 1996, Brady & Robins 2001). Their role in providing tissues with their biological properties has not been widely evaluated (Knott & Bailey 1998, Brady & Robins 2001).

The histidine adducts are the maturational products of the Lys^{ald} pathway. Trivalent histidinohydroxylysineonorleucine (HHL) is located in the C-terminal telopeptide. HHL has been found in skin (Yamauchi *et al.* 1987) and in cornea (Yamauchi *et al.* 1996). Some authors have suggested that ACP might react further with histidine resulting in the formation of a trivalent dehydro-

aldolhistidine, which then would react with Hyl to form a tetravalent dehydro-histidinohydroxylmerodesmosine (deH-HHMD) (Tanzer *et al.* 1973), but there is some doubt if their non-reduced forms actually function as cross-links (Robins 1988).

2.5.7 Methods for collagen cross-link measurement

The divalent cross-links have been studied by incorporating a tritium label into the reducible bond by reduction with $\text{NaB}[^3\text{H}]_4$. After this the tissue has been hydrolysed in acid to release the cross-links or an alkaline hydrolysis has been performed if the level of glycosylated cross-links has been studied. The hydrolyzed material is applied to an amino acid analyser and the tritium elution is monitored. This method provides the relative amounts of the different borohydride-reducible cross-linking residues within a collagen sample, but not the absolute concentrations per collagen content. Theoretically, absolute amounts can be calculated from the radioactivities, if the specific activity of the $\text{NaB}[^3\text{H}]_4$ is calibrated. (Eyre 1987)

The trivalent pyridinoline type cross-links have been measured from hydrolyzed tissue samples using reverse phase HPLC and the detection of the typical fluorescence patterns of these cross-links (Black *et al.* 1988). In order to quantify both the reducible and the mature cross-links in a single sample, ion-exchange chromatography and ninhydrin detection after initial removal of the conventional amino acid residues by molecular sieve chromatography has been used (Eyre 1987). Kinetics of cross-link formation have been studied using either ^{14}C or ^3H labelled lysine in tissue cultures or in animals *in vivo*, where the labelled lysine is incorporated to the cross-link (Eyre 1987). More recently immunochemical methods for the detection of pyridinoline type cross-links in biological fluids including urine (Robins *et al.* 1994, Vesper *et al.* 2002) serum, or sweat (Sarno *et al.* 1999) have been developed.

Pyrolic cross-links were first suggested in bone when a pink colour developed with the reaction of bone with p-dimethylaminobenzaldehyde and these were named Ehrlich chromogens (Scott *et al.* 1983). Later the Ehrlich-chromogen containing peptides were shown to derive from collagen using diazo-affinity columns to covalently bind these structures from bone and skin and these peptides were characterised by amino acid sequencing (Kuypers *et al.* 1992). Brady and Robins (2001) characterised these structures by developing a biotinylated Ehrlichs reagent using a monomeric avidin column.

2.6 Age related changes in collagen composition

The ECM in most tissues consists of network of different collagens. Their relative proportions as well as the organisation of the collagenous matrix and collagen cross-linking vary due to the continuous remodelling of the ECM in its different developmental states and with ageing of the tissue. In addition, progressive racemisation and isomerisation, deamination and non-enzymatic glycosylation of specific amino acid residues take place as the protein ages. In conjunction with age the rate of protein turnover has been shown to decline in rat heart (Crie *et al.* 1981) and in cultured human fibroblasts (Goldstein *et al.* 1976).

For reviews see Stadtman 1988, Bailey *et al.* 1998 and Robins & Brady 2002.

2.6.1 Changes in the enzymatic cross-links

The intermediate collagen cross-links mature into higher valency cross-links during ageing of the tissue (Avery & Bailey 2005). In human cortical bone, the amount of divalent cross-links (DHLNL+HLNL) has been reported to be about 2 mol/mol collagen in the first year after birth and gradually diminish to about half by the age of 25 years. (Eyre *et al.* 1988) With further reactions, they are considered to mature to more stable trivalent structures, but still remain the predominant cross-links in bone throughout the adult life (Eyre *et al.* 1988, Jonsson *et al.* 1985). The amount of pyridinoline type cross-links increases rapidly during the first two decades of life, but then remains constant through adult life (Oxlund *et al.* 1996, Eyre *et al.* 1988). The kinetics of the pyrrole type cross-links have not yet been described, but preliminary analyses have suggested that the pyrrole type cross-link content remains quite constant through adult life, but much higher concentrations are present in juvenile bone (Robins & Brady 2002).

The estimated half-life, based on [³H]lysine labelled rabbit cartilage, for the maturation of the divalent keto-imine cross-links to the mature trivalent structures has been reported to be about two weeks. In demineralised bone collagen, this maturation was found to be faster than in mineral protected collagen. This has led to the proposal that the mineral phase in bone would sterically hinder the maturation of the divalent cross-links to the trivalent forms. (Eyre *et al.* 1988) Others have suggested that the decrease in divalent cross-links in bone would be caused additionally by their dissociation due to mineral crystal formation and growth, thus resulting in the formation of free Lys and Hyl aldehydes not

involved in cross-links (Otsubo *et al.* 1992). Another explanation for the high prevalence of divalent cross-links in bone is the continual remodelling, resulting in a higher proportion of newly formed fibrils compared with soft tissues (Robins 1988).

2.6.2 Other changes in collagen structure

Racemisation, i.e. conversion of naturally occurring L-amino acids to the biologically rare D-isomers, and isomerisation, i.e. transfer of the peptide backbone from the α -carboxy group to a side-chain β -carboxy group in susceptible Asp or Asn residues, are associated with the aging of proteins. They are thought to be spontaneous non-enzymatic chemical reactions. An aspartyl-glycine -site located in C-terminal telopeptide of α 1(I)-chain (Fledelius *et al.* 1997) and in the N-terminal telopeptide of α 2(I)-chain (Brady & Robins 1999) have been documented to undergo this phenomenon. The formation of a succinimide intermediate leads to the development of the L- and D-isomers. A mixture of four isomers exists; the native peptide form (α L), an isomerised form containing a β -Asp bond (β L), a racemised form containing a D-Asp residue (α D) and an isomerised/racemised form (β L). In the C-terminal telopeptide of type I collagen, the ratio of α L to α D has proved to be most discriminatory in terms of indicating the biological age of the tissue or sample (Cloos & Fledelius 2000). This phenomenon has been used as a tool for examining the biological age of different tissues (Gineyts *et al.* 2000) and also in the assessment of fracture risk in osteoporotic women (Garnero *et al.* 2002) as well as the response to bisphosphonate treatment in patients with metabolic bone disease (Cloos *et al.* 2003). In children and patients suffering from Paget's disease of bone level of isomerised and/or racemised forms is lower indicating increased bone turnover rate (Garnero *et al.* 1997, Cloos *et al.* 2003). Patients suffering from osteogenesis imperfecta were recently found, in addition to having decreased type I collagen synthesis and increased degradation, also displayed a decreased isomerisation level of the C-telopeptide of type I collagen (Garnero *et al.* 2009).

2.7 Collagen degradation

Degradation of the ECM is of paramount importance in many physiological situations, such as morphogenesis, bone remodelling, angiogenesis and wound healing. It has a pathogenic role in many situations, such as tumorigenesis,

tumour invasion, Paget's disease of bone, rheumatoid arthritis and atherosclerosis. Many fibrotic situations involve impaired ECM degradation. It has been suggested that immediately following synthesis, up to 30% of newly formed collagen would not be secreted but degraded intracellularly. This might be a regulatory step inhibiting defective molecules from becoming attached to the fibrils. (Bienkowsky *et al.* 1978)

The proteolytic enzymes degrading ECM can be divided into groups of matrix metalloproteinases (MMP) and cysteine, aspartate and serine proteinases. When the ECM is degraded, two pathways are involved, the intra- or extracellular routes. Extracellularly collagens can be degraded by collagenases, gelatinases A and B, stromelysins and cathepsins. The portion of incompletely degraded material is phagocytosed by cells such as fibroblasts, macrophages and smooth muscle cells. Intracellularly these fragments are further broken down in the lysosomal apparatus (figure 3). (Everts *et al.* 1996, Berg *et al.* 1984)

2.7.1 Matrix metalloproteinases

The MMP's are a family of zinc-dependent neutral endopeptidases, which are capable of degrading essentially all of the ECM components. In physiological situations, the MMP activities are tightly regulated at the level of transcription, activation, interaction with specific ECM components and inhibition. To date, 24 vertebrate MMP's have been identified, of which 23 are found in humans. The MMP's are divided into subgroups of collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMP's and other MMP's.

Collagenases (MMP-1, MMP-8 and MMP-13 and MMP-18) are capable of degrading type I, II, III and V collagens in their fibrillar form, but have also other substrate specificities. They cleave the fibrillar collagens at specific sites (between helical residues 775 and 776 in type I collagen) generating N-terminal $\frac{3}{4}$ and C-terminal $\frac{1}{4}$ fragments. These denature at body temperature and can be further degraded by other proteolytic enzymes e.g. gelatinases. MMP-1 has the highest specificity for type I collagen, whereas MMP-8 degrades both type I and type III collagens. MMP-13 cleaves type II collagen in preference to type I or III collagen. MMP-13 can also cleave type I collagen at the N-terminal telopeptide domain. It has a much broader substrate specificity than the other collagenases and also has a higher gelatinase activity than MMP-1 or MMP-8.

Gelatinases (MMP-2 and MMP-9) have an important role in the final degradation of fibrillar collagens after the fibrillar organisation has been disrupted.

MMP-2 can cleave native type I collagen to similar fragments as collagenases, namely N-terminal $\frac{3}{4}$ and C-terminal $\frac{1}{4}$ fragments. MMP-9 cleaves type I, II and V collagens in the N-terminal telopeptide domain.

Stromelysin (MMP-3) activates a number of proMMP's and its activity is critical for the generation of fully active MMP-1. Membrane-type MMP's, with the exception of MT4-MMP, are responsible for the main activation of proMMP-2. MT1-MMP has collagenolytic activity on type I, II and III collagens.

Tissue inhibitors of matrix metalloproteinases (TIMP) are specific inhibitors that bind MMP's with a 1:1 stoichiometry. At present, four TIMP's (TIMP1-4) have been identified in vertebrates. TIMPs inhibit all MMPs tested so far, except that TIMP-1 does not inhibit MT1-MMP. The α -macroglobulin is also able to inhibit MMPs.

For reviews see Nagase & Woessner 1999, Kähäri & Saarialho-Kere 1999, Delaissé *et al.* 2000, Hojilla *et al.* 2003, Visse & Nagase 2003, Nagase *et al.* 2006.

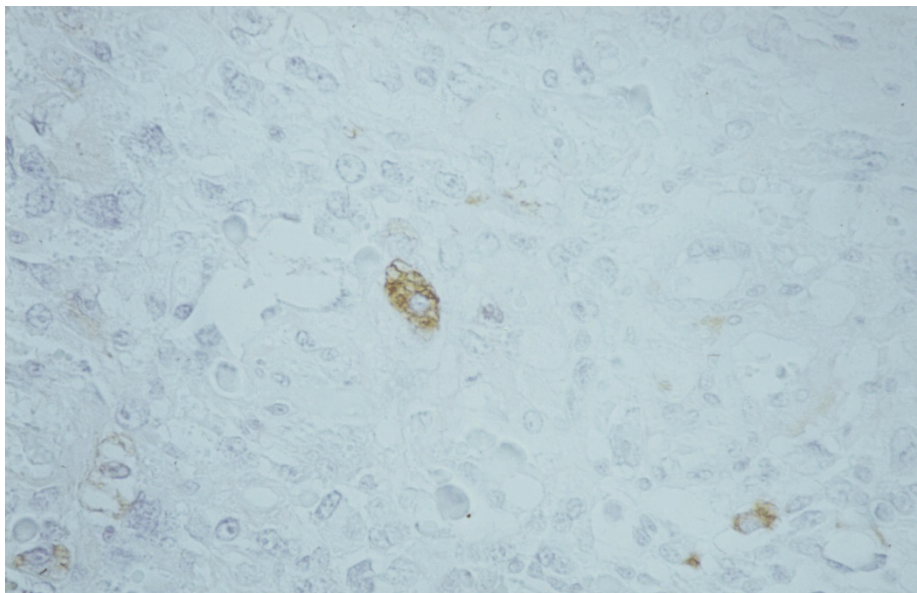


Fig. 3. Immunohistochemical staining with purified ICTP antibody of the stroma of mixed müllerian tumour shows intracellular staining with ICTP antibody suggesting intracellular degradation by macrophages. Photographed by Salla Kauppila.

2.7.2 Cysteine, serine and aspartate proteinases

Lysosomal cysteine proteinases, commonly called the cathepsins, are papain-like enzymes optimally active in slightly acidic, reducing milieu. These enzymes are able to degrade ECM both extra- and intracellularly. Cathepsins have been associated with number of pathological events such as rheumatoid arthritis, osteoarthritis, cancer, neurological disorders, osteoporosis and lysosomal storage diseases. Cathepsins also participate in apoptosis (Salvesen 2001).

Cathepsin K has been found to be expressed almost exclusively in osteoclasts (Lecaille *et al.* 2008). In osteoclasts isolated from giant cell tumour of bone, cathepsin K was expressed 20-, 130- and 410-fold stronger than cathepsins B, L and S, respectively (Ishibashi *et al.* 2001). It is able to cleave native triple helical type I and II collagens at multiple helical sites and telopeptides (Garnero *et al.* 1998, Kafienah *et al.* 1998a). The collagenolytic activity requires the formation of a complex between cathepsin K and specific glycosaminoglycans (GAG) (Li *et al.* 2002) pointing to a regulatory effect of GAGs in cathepsin K mediated

degradation between tissues (Li *et al.* 2004). Other tissues with a lower degree of cathepsin K expression include the human breast carcinomas and various breast cancer cell lines (Littlewood-Evans *et al.* 1997), ovary (Brömme & Okamoto 1995), thyroid (Teipel *et al.* 2000) and lung (Bühling *et al.* 2004). Loss of function of cathepsin K leads to pycnodysostosis, characterised by short stature, osteopetrosis, acro-osteolysis of the distal phalanges, frequent fractures, clavicular dysplasia and skull deformities with delayed suture closure (Gelb *et al.* 1996). Cathepsin S is an active lysosomal enzyme being capable of degrading fibrillar collagens. Cathepsin L is found throughout all mammalian cell types, most crucial functions being assigned to its role in immune and the central nervous system. It has been thought to have a pathogenetic role in situations such as bone resorption, tumour metastases and rheumatoid arthritis. A serine protease, human neutrophil serine elastase, has collagenolytic activity towards type I collagen (Kafienah *et al.* 1998b).

Natural inhibitors of the cathepsins include the proregions of the corresponding proteinases, inhibitors of the cystatin family (cystatins, stefins and the related kininogens) and the recently characterised serpin.

For reviews see McGrath 1999, Turk *et al.* 2000, Wolters & Chapman 2000, Turk *et al.* 2001, Brix *et al.* 2008.

2.8 Assessment of collagen metabolism

Markers of collagen metabolism have become valuable clinical tools for the diagnosis and follow-up of several diseases, including rheumatoid arthritis, osteoporosis, Paget's disease of bone and liver cirrhosis. They have prognostic values in cancers of breast (Keskikuru *et al.* 2002, Jukkola *et al.* 2001), ovary (Santala *et al.* 1999), prostate (Jung *et al.* 2004) and multiple myeloma (Jakob *et al.* 2008). They have also been utilised to investigate the impact of nutrition and physical activity on bone metabolism (Branca *et al.* 1992, Sorva *et al.* 1994, Sharp *et al.* 1997).

The aminoterminal (PINP and PIIINP) and carboxyterminal (PICP and PIIICP) propeptides of type I and III collagens, respectively, are removed from the newly formed collagen molecule. The intact PINP, PICP and PIIINP assays measured in serum (Niemelä *et al.* 1985, Melkko *et al.* 1990, Melkko *et al.* 1996) or salt extracts of the tissues (Mansell & Bailey 1998, Bode *et al.* 1999) have been suggested to be good markers of collagen synthesis.

The oldest collagen degradation marker is the urinary hydroxyproline measurement. However, diet and other proteins with hydroxyproline in their structure can affect the results. The more recently developed collagen degradation assays are generally based on the assay of stable trivalent pyridinoline type cross-links (Robins *et al.* 1994) or on the cross-linked telopeptide structure measurements (ICTP (Risteli *et al.* 1993), NTx (Hanson *et al.* 1992) and CTx (Bonde *et al.* 1995)) either from serum or urine. Unlike the pyridinoline type cross-link assays, the telopeptide structure based assays are collagen type specific. The epitopes of NTx and CTx assays are produced by cathepsin K mediated degradation (Nishi *et al.* 1999, Atley *et al.* 2000, Garnero *et al.* 2003). ICTP, on the other hand, is liberated by MMP-9 mediated degradation, allowing these assays to reflect different collagen degradation pathways (Garnero *et al.* 2003).

For review see Risteli & Risteli 2006.

2.9 Bone

Bone is comprised of a hydroxyapatite mineral and an organic matrix, which is mostly (over 90%) type I collagen. About 75% of the mineral present in bone is located within the collagen fibrils. (Katz *et al.* 1989) Bone consists of cortical and trabecular compartments which differ, not only in their function, but also in their composition. Trabecular bone has been reported to have higher bone alkaline phosphatase activity than cortical bone. On the other hand, the osteocalcin content has been found to be 3-fold higher in cortical bone compared to trabecular bone. (Magnusson *et al.* 1999) About 80% of bone is comprised of cortical bone and only about 20% of trabecular bone. Despite this, the trabecular bone has a much higher overall surface area compared with cortical bone, which is essential for the metabolically active trabecular bone. (Ott 1996) For example, the bone volume in the trabecular bone of vertebrae is only 7–14% of the total, the rest being bone marrow.

Remodelling of bone is crucial for the maintenance of bone mass, skeletal integrity and function. It is based on the co-ordinated function of the osteoclasts and osteoblasts. Osteoclasts dissolve the mineral by producing an acidic environment and then the organic matrix is broken down by proteolytic enzymes. Soon after the bone resorption, the osteoblasts invade the area and start to produce new bone matrix called osteoid. The osteoid is composed mainly of type I collagen, with a variety of proteoglycans (for example decorin and biglycan), glycoproteins (for example osteonectin), RGD-containing glycoproteins (in

addition to type I collagen for example osteopontin and bone sialoprotein) and γ -carboxylated proteins (for example matrix-gla-protein (MGP) and osteocalcin). For reviews see Pogoda *et al* 2005, Tanaka *et al.* 2005.

Appropriate mineralization depends on a correct alignment of collagen molecules in the fibril, because the nucleation of calcium apatite crystals begins in the gap region (Yamauchi & Katz 1993). The collagenous matrix must undergo a maturation process before it can support mineral induction and growth. Osteoblasts are required for this maturation process, but not for the mineralisation of the mature bone-like extracellular matrix (Marsh *et al.* 1995). Another study postulated that the mineralisation of bone can occur in the absence of cells since foetal bovine serum was seen to induce mineralization of demineralised bone or tendon tissue in cell culture conditions, both of which are primarily composed of type I collagen. The authors suggested that serum contains some kind of nucleator of the type I collagen matrix mineralization. (Hamlin *et al.* 2004)

Collagen cross-linking in bone differs from that in soft tissues (see section 2.5). The proper organisation and cross-linking of collagen provides the bone with mechanical properties (Boskey *et al.* 1999). When rats were treated with β -aminopropionitrile, a substance that irreversibly inhibits the enzyme lysyl oxidase, a 45% reduction in trivalent HP cross-links and a 31% decrease in the stability of bone collagen towards acetic acid and pepsin treatment was observed, compared to control rats (Oxlund *et al.* 1995). The trabecular bone has a lower pyridinoline type cross-link content compared to cortical bone, presumably reflecting the higher turnover rate (Eyre *et al.* 1988, Noris Suarez *et al.* 1995). In trabecular bone of the iliac crest, the collagen content has been reported to decrease with age (Bailey *et al.* 1999). A small study revealed a relationship between the nature of the collagenous matrix, particularly the ratio of trivalent pyrrole to HP cross-links, and the structural organisation of the trabeculae in vertebral trabecular bone. Samples with high pyrrole concentration had relatively thicker trabeculae, which were less numerous and less structurally complex. (Banse *et al.* 2002a) Another work indicated that vertebral bone with a higher HP to LP ratio would be stronger and stiffer (Banse *et al.* 2002b). The amount of divalent DHLNL and HLNL cross-links has been shown to be decreased by 30% and 24% in osteoporotic trabecular bone (Oxlund *et al.* 1996). The divalent DHLNL cross-link content was diminished by 20% in the osteoporotic femoral head and 44% in the femoral neck compared with controls (Bailey *et al.* 1993). Neither of these studies found differences in the contents of pyridinoline type cross-links between osteoporotic and control bone samples.

2.10 Ectopic calcification

Calcification has been described as being pathological when taking place in functional soft tissues, and physiological when occurring in skeletal tissues. Ectopic calcification is defined as inappropriate biomineralisation in soft tissues (Cotran *et al.* 1994). When ectopic calcification occurs in the absence of any systemic mineral imbalance, it is termed dystrophic calcification (Giachelli 1999). Dystrophic calcification may be often detected in injured, diseased and aged tissues, showing evidence of tissue alteration or even necrosis. Skin, kidney, tendons, and cardiovascular tissues are particularly susceptible to dystrophic calcification, although most tissues can undergo calcification (Anderson *et al.* 1993). There are a few disorders where ectopic calcification or bone formation occurs extraskeletally, for example dermatomyositis and fibrodysplasia ossificans progressiva.

2.10.1 Calcification of vascular tissues

Dystrophic or ectopic calcification occurs in numerous cardiovascular conditions including atherosclerosis, valvular stenosis, and reperfused ischemic myocardium (Srivatsa *et al.* 1997). It has long been considered as an end-stage passive mineral precipitation induced by inflammatory factors and it is often associated with advanced tissue degeneration or even necrosis. The mechanism of passive precipitation postulates that it is only the presence of inhibitors under homeostatic conditions that prevent calcium from precipitating. Apoptotic cell bodies together with cholesterol can function as a nidus, where mineralisation can occur if local ionic concentrations exceed the salt solubility product. However, during the past decade, many studies have viewed atherosclerosis and aortic valve calcification as an active, complex and presumably a regulated process that exhibits many similarities to embryonic bone formation and bone remodelling (Hunt *et al.* 2002). See Barnes & Farndale 1999, Farzaneh-Far *et al.* 2001, Doherty *et al.* 2003, Vattikuti & Towler 2004, for reviews.

The process of atherosclerosis formation as well as valve calcification starts with endothelial damage. In the calcific aortic valves, the lesion often resides in the aortic rather than ventricular side of the valve. Vascular injury in response to atherosclerotic risk factors promotes endothelial dysfunction. This results in inflammation, evidenced by the presence of T-lymphocytes even in the early lesions. Adhesion molecules recruit the monocytes into the subendothelial space,

where they differentiate into macrophages. Macrophages accumulate lipid through membrane scavenger receptors, which are able to bind oxidised LDL cholesterol, resulting in foam cells. Monocytes and macrophages together with lipids have been found to colocalise with calcium deposits in plaques (Hirsch *et al.* 1993, Jeziorska *et al.* 1998b) suggesting that the lipid components in atherosclerotic plaque may serve as the nidus for mineralisation. The observation of positive correlations between postmenopausal osteoporosis and atherosclerotic calcification led to the proposal that oxidised LDL cholesterol can upregulate the osteogenic differentiation and mineralisation of the calcifying vascular cells, but with opposing effects on bone cells (Parhami *et al.* 1997, Tintut *et al.* 2002, Proudfoot *et al.* 2002). Monocytes are also able to enhance the osteoblastic differentiation of calcifying vascular cells, as evidenced by increased alkaline phosphatase activity and increased matrix mineralisation (Tintut *et al.* 2002). Lamellar bone formation has been found to occur in 13% of carotic artery plaques (Jeziorska *et al.* 1998a, Hunt *et al.* 2002).

2.10.2 Calcific aortic valve stenosis

Non-rheumatic, calcific aortic valve stenosis (AS) is the most common heart valve disease and the main cause for heart valve replacement in the elderly. Aortic valve stenosis shares many risk factors with atherosclerosis, namely age, hypertension, hypercholesterolemia and cigarette smoking, and it has been claimed to resemble atherosclerosis in many ways. (Rajamannan *et al.* 2003) During recent years, it has also been thought to be an actively regulated phenomenon, resembling bone formation in several aspects (see Farzaneh-Far *et al.* 2000, Mohler 2004, for reviews).

Histopathologically AS is characterised by fibrous thickening of valve leaflets with an extensive remodelling of the ECM and focal calcification (Otto *et al.* 1994). Cultured myofibroblasts from cardiac valves can undergo phenotypic differentiation into osteoblast-like cells (Mohler 2004). The matrix of calcified aortic valves contains many bone matrix proteins, such as type I collagen, osteopontin, osteonectin, MGP and bone morphogenetic proteins (BMP) (Mohler *et al.* 1997, Srivatsa *et al.* 1997). The inhibitors of calcification, namely MGP, osteocalcin and a potential inhibitor, bone sialoprotein, are expressed at all stages of the human atherosclerotic process, indicating a contiguous inhibition of calcification in the atherosclerotic vessel wall. Proteins known to activate calcification, namely BMP-2, BMP-4, osteopontin and osteonectin, have been

found only in advanced and calcified lesions. (Dhore *et al.* 2001) Recently, the receptor activator of nuclear factor κ B ligand (RANKL) and osteoprotegerin have been found to be expressed in calcified aortic valves; they have been proposed to function as regulators of calcification. RANKL induces matrix calcification and transition of the cultured human aortic valve myofibroblasts towards an osteogenic phenotype. Osteoprotegerin is highly expressed in normal aortic valves whereas stenotic valves display lower amounts. (Kaden *et al.* 2004) Statins inhibit calcification in cultured aortic valve myofibroblasts and inhibit the alkaline phosphatase production of these cells through inhibition of the cholesterol synthesis pathway (Wu *et al.* 2005). Paradoxically statins have been shown to induce bone formation in rodents by stimulating osteoblastic differentiation and bone cell mediated calcification by acting on BMP-2 promoter (Mundy *et al.* 1999, Wu *et al.* 2005). Mohler *et al.* (2001) found 13% of calcified valves with end-stage valvular heart disease to have mature lamellar bone with hematopoietic elements and active bone remodelling.

The collagen cross-linking in human aortic valves has not been extensively studied. Balguid *et al.* (2007) compared the collagen cross-link content between native healthy human aortic valves, mean age 48.9 years, to that of tissue engineered ones. The HP cross-link was the only measured cross-link and was found to be 0.26 ± 0.05 mol/triple helix (Balguid *et al.* 2007). A recent study of collagen cross-linking of the bovine heart valves (aged 24 to 30 months) implied that all valves contained more ketoimine derived cross-links than aldimine derived cross-links. None of the valves had measurable amounts of LP. HP was found as the major cross-link, with the aortic valve containing 0.709 ± 0.038 mol/mol of collagen. The HHL cross-link content was 0.026 ± 0.004 mol/mol of collagen. However, the valves with higher HHL amounts were found to be hydrothermally more stable. (Aldous *et al.* 2009) Little is known about the changes in the collagen matrix of human aortic valves in health and disease.

2.11 Tendon

Tendons are force-transmitting tissues primarily composed of a dense network of type I collagen fibrils. Type I collagen constitutes around 60–70% of the dry mass and approximately 95% of the total collagen in healthy tendon tissue. Type III collagen accounts for about 3% of the total collagens in human supraspinatus and biceps brachii tendons. Tendons also contain considerable amounts of

proteoglycans and glycoproteins, such as decorin, biglycan, lumican, fibromodulin, versican and aggrecan. (Riley 2004, Kjær 2004)

The smallest structural unit in tendons is the fibril, which ranges from 10 to 500 nm in diameter. Fibrils assemble into bundles called fascicles, which are surrounded by endotenon. The endotenon carries blood vessels, lymphatics and nerves. Bundles of fascicles are surrounded by epitenon, a structure contiguous with the endotenon. The endo- and epitenon are thought to contain most of the type III collagen found in healthy tendon tissues, but with increasing age, type III collagen may also form heterotypic fibrils with type I collagen. The majority of the fibre bundles are aligned with the long axis of the tendon and are responsible for the tensile strength of the tissue. Transverse, spiral and plait-like alignments provide resistance against transverse, shear and rotational forces acting on tendon.

Collagen cross-linking provides the tendon with tensile strength as shown in a study, where stabilisation of divalent cross-links prior to acid treatment (which is known to disrupt some of the divalent cross-links, see section 2.5) increased the tensile strength of the rat tail tendon (Davison 1989). In healing rabbit medial collateral ligament, scar weakness was associated with decreased pyridinoline-type cross-link density (Frank *et al.* 1995). The trivalent HP cross-link levels vary significantly between tendons, the normal supraspinatus tendon having 0.80 and biceps brachii tendon 0.25 mol/mol of collagen, reflecting the different mechanical loading of the tendons (Bank *et al.* 1999b). Region-specific differences have also been shown to exist in the cross-link contents within the same tendon. The HP and LP cross-link contents were found to be higher in the posterior part of human patellar tendon than in the anterior part (1.416 ± 0.25 vs. 0.859 ± 0.197 and 0.035 ± 0.006 vs 0.023 ± 0.006 mol/mol of collagen). Regional differences in pyrrole concentrations were not found. (Hansen *et al.* 2010) In human Achilles tendon, the HP content has been reported to be highest at the age of 20 and to slightly decrease to the age of 60 (Moriguchi & Fujimoto 1978). A decreased HP content, together with decreased collagen content and increased solubility of collagen, has been reported also in tenotomised healing rabbit Achilles tendons compared to the control tendons. The tensile strength and elasticity of the healing tendons was also found to be decreased. (Reddy *et al.* 1999) In addition, the tendon tissue in supraspinatus tendinopathy was reported to relatively more type III collagen when compared to type I collagen (Riley *et al.* 1994a).

2.11.1 “Spontaneous” Achilles tendon rupture

The incidence of Achilles tendon ruptures has dramatically risen during the past few decades. Achilles tendon rupture occurs most frequently in men (the male: female ratio being approximately 6:1) aged 30 to 40 years doing recreational sports. Four of the every five of the ruptures are located 2 to 6 cm above the calcaneal insertion, where the vasculature is most restricted. (Leppilahti & Orava 1998)

There are two theories to explain the pathogenesis of spontaneous Achilles tendon rupture. Firstly, chronic degeneration of the tendon due to hypovascularity has been thought to weaken the tendon so that weaker mechanical loading can cause a total rupture. The other theory points to a failure in the inhibitory mechanisms of the musculotendinous unit. These mechanisms, however, may contribute mutually to the total Achilles tendon rupture.

Exercise increases type I collagen turnover in human Achilles tendon (Langberg *et al.* 1999). When performed continuously, early in the process both the synthesis and degradation of type I collagen are increased, but after 11 weeks of training, mainly anabolic effects are present causing a net synthesis of type I collagen (Langberg *et al.* 2001). However, overuse of the tendon (when the load exceeds the strength of the tissue) is thought to cause repetitive microtraumas, in which the regenerative processes are unable to keep in pace due to the low vasculature (Kjær 2004). Degenerative changes such as hypoxic changes, abnormal orientation and size of collagen fibers and mucoid type degeneration have been observed preceding rupture of Achilles tendon (Jozsa & Kannus 1997, Kannus & Jozsa 1991, Thermann *et al.* 2002, Kjær 2004) and are well recognised as predisposing factors for tendon rupture. In a large histochemical study, hypoxic changes were the most common type of degenerative changes (Kannus & Jozsa 1991). It has been reported that ruptured Achilles tendons contain increased type III collagen levels (Clement *et al.* 1984, Jozsa & Kannus 1997) and smaller diameter fibrils (Magnusson *et al.* 2002), which are thought to reduce the mechanical properties of the tendon. Cultured tenocytes from ruptured Achilles tendons produced more type III collagen than normal tenocytes (Maffulli *et al.* 2000). The turnover of the ECM was increased in tendinopathic Achilles tendons. In a study using cDNA arrays, an increase in the expression of type I and type III collagen, versican, biglycan and perlecan genes was reported. The same authors reported the absence of inflammation in tendinopathic Achilles tendons. (Ireland *et al.* 2001)

In a small number of individuals, however, Achilles tendon rupture may occur without any histological evidence of tissue degeneration. The fact that it was possible to induce Achilles tendon rupture in rats by rapid lengthening of the tissue, especially after a period of inactivity or when the involved muscle was fatigued, has supported the trauma theory. However, such mechanism may occur in healthy young athletes only during extreme physical activity. (Jozsa & Kannus 1997)

Most of the Achilles tendon ruptures occur during sports activities (60–75%) (Kannus & Natri 1997). The use of androgenic anabolic steroids concurrent with exercise has been documented to have adverse effects on tendons in both humans and rats. Stiffer tendons that absorb less energy and fail with less elongation have been found in rats subjected to the combination of anabolic steroid administration and exercise (Inhofe *et al.* 1995). The elevation in the MMP-2 activity caused by training has been shown to be inhibited by anabolic steroid administration in rat Achilles tendons leading to accumulation of disorganised collagen fibrils on the surface of tendon (Marqueti *et al.* 2006). In humans, it has been proposed that high doses of anabolic steroids decrease the degradation and might increase the synthesis of type I collagen. They have also been claimed to increase the synthesis of type III collagen in humans. (Pärssinen *et al.* 2000)

3 Aims of the present study

Proper type I collagen organisation and cross-linking are crucial for tissues optimal function. Collagen cross-linking has traditionally been studied using chemical cross-link analyses. However, these do not differentiate between genetically distinct collagen types or between the location of the cross-link at either the N- or C-terminal telopeptide. This study was performed to study cross-linking at the C-terminal telopeptide of type I collagen. In order to achieve this goal, an immunoassay was developed capable of detecting the divalently (“immature”) cross-linked structures originating from the C-telopeptide of type I collagen. By combining this with the earlier developed ICTP assay, different physiological and pathological tissues have been studied to gain information of cross-linking at C-telopeptide of type I collagen in various tissues. Achilles tendon and calcified aortic valves were studied also to determine the type III collagen composition.

The specific aims of this study were:

1. To isolate and characterise the differently cross-linked and non-cross-linked carboxyterminal telopeptide structures of human mineralised bone.
2. To measure the concentration of ICTP like structures in human cortical and trabecular bone.
3. To characterise the epitope of the ICTP assay.
4. To investigate the collagenous matrix in ruptured and normal Achilles tendons.
5. To investigate what kind of changes in the collagenous matrix occur with aortic valve calcification.

4 Materials and methods

4.1 Tissue samples (I-IV)

Human bone was obtained from routine hip replacement operations in the Department of Surgery, Oulu University Hospital. The femoral heads obtained were usually either osteoarthrotic or osteoporotic (or both) and were stored at $-20\text{ }^{\circ}\text{C}$ until used.

Healthy human bone ($n = 5$) was obtained from a tissue bank. None of the donors had a disease or were taking drugs known to affect bone metabolism. The cortical bone was cut from the periosteal surface of proximal diaphysis of the femur and cubes of trabecular bone were removed from the trochanteric region.

Human Achilles tendon samples were obtained during operation from 10 individuals with total Achilles tendon rupture. The patients were operated using the method described by Sifverskjöld, where a flap from the gastrocnemius tendon was turned above the rupture site (Silfverskiöld 1941). Small tissue samples from three different sites of the tendons were dissected; one from the rupture site (approximately 4 cm from the calcaneal insertion), one from the lower end (approximately 8 cm from calcaneal insertion) and one from the top corner (16 cm from the calcaneal insertion) of the flap. The control Achilles tendon samples were taken from six cadavers, who did not have any known diseases nor were taking any drugs affecting the tendon. The cadaver tendons were taken within 72 hours post-mortem. Tissue samples from the three corresponding sites were dissected from the cadavers. The tissue samples were stored at $-70\text{ }^{\circ}\text{C}$ until used.

Calcified human aortic valves ($n = 11$) were obtained from routine valve replacement operations from the Department of Surgery, Oulu University Hospital. Control aortic valves were obtained from cadavers of different ages (under 50 years old, $n = 14$, those over 50 years old $n = 10$) from the Department of Forensic Medicine, Oulu University. The samples were stored at $-70\text{ }^{\circ}\text{C}$ until used.

4.2 Assay for the synthetic peptide SP 4

A synthetic peptide of 17 amino acids (Ser-Ala-Gly-Phe-Asp-Phe-Ser-Phe-Leu-Pro-Gln-Pro-Pro-Gln-Glu-Lys-Tyr, M_r 1958), analogous to the non-helical

carboxyterminal telopeptide region of the $\alpha 1$ chain of human type I collagen, was commercially synthesised (Neosystem Laboratories, Strasbourg, France) and named SP 4. An extra tyrosine residue was included at the C-terminus of the peptide to facilitate radioiodination. The SP 4 peptide was used as a reference inhibitor to construct the standard curve. Three μg of the peptide was also radioiodinated by the chloramine T method. A polyclonal antiserum (rabbit number 176), previously raised to a highly purified preparation of human trivalently cross-linked ICTP antigen, was selected for use in the immunoassay for SP 4.

Aliquots of the SP 4 standard or the unknown sample (100 μl) were incubated with 200 μl of iodinated SP 4 tracer (equivalent to 50 000 cpm) and 200 μl of antiserum (diluted 1:1500) for 2 hour (+37 °C) and then 500 μl of the second antibody-PEG solution was added (goat anti-rabbit gammaglobulin (1:50) and 15% PEG in 0.1 M phosphate buffer, pH 7.5, containing 0.05% Tween 20), and incubated for 30 min (+4 °C). The bound fraction was separated by centrifugation (2000 \times g for 30 min at +4 °C). Supernatants were decanted and the precipitates counted using a Wizard™ 1470 automatic gamma counter (Wallac, Turku, Finland).

The abilities of the SP 4 peptide and other type I collagen $\alpha 1$ -chain carboxyterminal telopeptide structures to compete with iodinated SP 4 for binding to the antiserum were compared using a quantitative immuno-inhibition analysis procedure. This estimated the slopes and 50% intercepts of the inhibition curves (Risteli & Risteli 1987). The antigens tested included highly purified trivalent ICTP, the divalent $\alpha 1_{\text{C}}\alpha 1_{\text{H}}$ and $\alpha 1_{\text{C}}\alpha 2_{\text{H}}$ structures and the uncross-linked free $\alpha 1_{\text{C}}$ telopeptide. These all were purified from human bone. For comparison, the trivalent histidinohydroxylysinoxorleucine (HHL) cross-linked C-telopeptide of type I collagen ($\alpha 1_{\text{C}}\alpha 2_{\text{H}}\alpha 1_{\text{H}}$) purified from human skin, was also tested.

4.3 Preparation of the bone and isolation of the carboxyterminal telopeptide structures of type I collagen from human bone (I, II)

The human femoral heads were cut into small pieces, frozen in liquid nitrogen and powdered in a mineral mill (Retsch AG, Haan, Germany). The fat was extracted from the bone powder by acetone/methanol or chloroform/methanol, centrifuged (15000 \times g for 30 min) and air-dried. The bone powder was then reduced with either NaBH_4 or $\text{NaB}[^3\text{H}]_4$ as follows: The bone powder was suspended in PBS, pH 7.2, in concentration of 100 mg/mL. The NaBH_4 solution was prepared just prior to use in 1 mM NaOH (concentration 25 mg/ml). One mg

of NaBH₄ was added per 40 mg of tissue in the solution and incubated in magnetic stirring for two hours at room temperature. Subsequently the residue was washed several times with distilled water, centrifuged and decanted. The healthy bone samples were treated similarly.

After reduction with NaBH₄, 300 mg of homogenic bone powder was suspended in 3 ml of PBS-tween 20 (0.05%). The extraction of bone powders was carried out in water bath overnight in constant shaking at +37 °C and the suspensions were centrifuged on the next day at 10 000 rpm for 30 min at +4 °C. The supernatants were collected and assayed for total PINP and PICP.

Soft connective tissue outside the mineral was then removed by trypsin digestion (described in detail below). This fraction, called the SOFT fraction, was stored frozen for further analyses. Mineralised bone powder was demineralised with 0.5 M EDTA, 50 mM Tris-HCl, pH 7.4, in the presence of 1 mM cathepsin K inhibitor E64 (*trans*-epoxysuccinyl-L-leucylamido[4-guanidino]-butane) (Sigma, St. Louis, MO, USA), for four days at +4 °C with three changes of the demineralisation solution. After washing several times with distilled water, the bone collagen was then heat denatured (+65 °C for 30 min), cooled to +37 °C and digested with TPCK (N-tosyl-L-phenylalanine chloromethyl ketone)-treated trypsin (Worthington Biochemicals, Lakewood, NJ, USA) (enzyme substrate ratio 1:50) in the presence of additional TPCK (Sigma, St. Louis, MO, USA) (1 mg/4 mg of trypsin) for six hours. The digest was heat denatured again and the trypsin digestion was repeated overnight. A final heat denaturation was performed on the next day to inactivate the residual trypsin activity and the digests were centrifuged (15000 × g for 30 min). The supernatant was taken for further analyses.

The lyophilised crude tryptic digest of demineralised bone matrix was initially applied in batches of 1 g/100 ml to a preconditioned preparative Sep-Pak C₁₈ (Millipore Waters, Milford, MA, USA) solid phase extraction column and washed with 100 ml of 30% methanol. The α1(I)-chain telopeptides were eluted with 100 ml of 70% methanol and lyophilised. A 300-mg aliquot of the concentrated telopeptides was applied onto a size exclusion chromatography (SEC) column (130 × 2.5 cm) of Sephacryl S-100 HR (Pharmacia, Uppsala, Sweden) on a flow rate of 20 ml/h collecting 15 min fractions. The fractions were analysed for ICTP and SP 4. The pooled lyophilised fractions containing SP 4 immunoreactive antigens were further purified by C₈ (228TP1010, Vydac, Hesperia, CA, U.S.A) reverse phase HPLC using 0.4% ammonium acetate, pH 7.4, as buffer A and eluting the peptides with increasing gradient of 75% acetonitrile in buffer A. Further purification of the C-telopeptide components was

achieved with a C₁₈ (218TP1010, Vydac) HPLC reverse phase step in 0.1% TFA (buffer A) and eluting with a gradient of 70% isopropanol (buffer B). A DEAE anion exchange (Protein Pak DEAE 5 PW, Millipore Waters) HPLC step, in which elution was carried out with an ammonium acetate gradient (from 0.02 to 0.4 M) at pH 7.4 containing 1% of 2-propanol, was finally performed. At all steps, the elution of the peptides was followed with absorbance (280 nm), fluorescence measurements (excitation 295 nm in TFA and 320 nm in ammonium acetate, emission 395 nm and 405 nm, respectively) and by the SP 4 and ICTP assays.

4.4 Characterisation of the purified peptides (I, II)

The identities of the purified carboxyterminal telopeptide structures were confirmed by N-terminal sequencing (Procise 492, Applied Biosystems, Foster City, CA, U.S.A.). Their molecular weights were determined by MALDI-TOF MS (Biflex™, Bruker-Franzen Analytic) (Saarinen *et al.* 1999). Amino acid analysis (Biochrom 20 amino acid analyser, Pharmacia, Cambridge, England) of the purified peptides was performed after acid hydrolysis by loading 70 µl of sample in lithium citrate loading buffer, pH 2.2.

The chromatographically purified peptides were dissolved in NuPAGE LDS sample buffer (Invitrogen, Frankfurt, Germany) and electrophoresed on 4–12% NuPAGE Bis-Tris gels (Invitrogen) using NuPAGE MES SDS (Invitrogen) running buffer system. Protein bands were visualised with Colloidal Blue stain (Invitrogen).

4.5 Production of polyclonal antibodies

Thyreoglobulin conjugated human ICTP or SP 4 peptide was given to New Zealand White rabbits to evoke the production of polyclonal antibodies. A mixture of 250–1000 nmol of peptide and 10 mg of bovine thyreoglobulin (MW 660 000) was suspended to 500 µL of 10 mM NaHPO₄ buffer. One hundred mg of carbodi-imide (1-ethyl-3-(3-dimethylaminopropyl) (Sigma) suspended in 1 mL of 10 mM NaHPO₄ buffer was added to the peptide-thyreoglobulin mixture and incubated in ice for 2 h with constant stirring. Finally the mixture was dialysed against PBS, pH 7.2 overnight. 100–200 µL of conjugated antigen, ad 1 mL of 0.9% NaCl was mixed into an emulsion with 1 mL either complete (first immunisation) or incomplete (boosters) Freund's adjuvant (Sigma) and injected intradermally at three to four weeks intervals.

4.6 Preparation of the analytical samples (III, IV)

The Achilles tendon (III) and aortic valve (IV) samples were weighed, cut into pieces, suspended in PBS, pH 7.2 in a concentration of 20–100 mg/ml and homogenised by sonication in an ice bath. The samples were incubated for 30 min and centrifuged (15000 g for 30 min) to separate the extracted soluble tissue from the insoluble material. The concentrations of PINP, PICP and PIIINP as well as total protein were measured from the soluble tissue extracts.

The insoluble tissue residues were then suspended again in PBS, pH 7.2, and reduced with NaBH₄. After thorough washing, the tissue pieces were lyophilised and weighed. The Achilles tendon samples were then digested with trypsin as described above. The concentrations of hydroxyproline, ICTP, SP 4, IIINTP and tryptic PIIINP were measured from the digests.

The control aortic valves were treated similarly to the Achilles tendon samples. From the calcified aortic valves, the collagen fraction outside the mineral was removed by trypsin digestion. The remaining mineral protected tissue was demineralised using 0.5 M EDTA, 50 mM Tris-HCl, pH 7.4, for two days with two changes of the demineralisation solution. Then it was lyophilised and weighed. Finally the exposed mineral protected matrix was digested with trypsin. The total protein content, and the levels of hydroxyproline, ICTP, SP4, IIINTP and tryptic PIIINP were measured from both soft connective tissue and mineralised fractions of the calcified aortic valves.

4.7 Total protein, hydroxyproline and different collagen antigen measurements

Aliquots of the tryptic digests were hydrolysed in 6 M HCl at 106 °C for 16 h. Hydroxyproline was measured in the tissue hydrolysates using a colorimetric method adapted for a microtitre-plate format (Brown *et al.* 2001). Total tissue collagen was calculated assuming that hydroxyproline accounts for 12.4% (w/w) of the total collagen. The total protein content was measured by the Bio-Rad DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

4.8 Immunohistochemical staining of the collagen antigens (IV)

The purified antibodies for PINP, PIIINP, ICTP and IIINTP were used for the immunohistochemical staining of the human aortic valves. In the immunostaining, the avidin-biotin-peroxidase method was used. Five μm sections were deparaffinised and incubated in a solution containing 0.14 g trypsin in 100 ml of 0.1% CaCl_2 pH 7.8 for 40 minutes at +37 °C. After the trypsin pretreatment, the slides were washed in PBS and specific rabbit anti-human antibodies against the PINP (dilution 1:100), PICP (1:10), ICTP (1:10), PIIINP (1:10) and IIINTP (1:50) were applied on the slides (Bode *et al.* 2000). This was followed by the biotinylated secondary anti-rabbit antibody and the avidin-biotin-peroxidase complex. The colour reaction was developed by diaminobenzidine. As a negative control PBS was substituted for the primary antibody in the staining reaction. The staining reaction was semiquantitatively divided into four groups: - = no staining, + = weak staining (0–10% of valvular area positive), ++ = moderate staining (10–20% of valvular area positive), and +++ = strong staining (> 20% of valvular area positive).

4.9 *In situ* hybridisation (IV)

A 400 bp cDNA fragment from the carboxyterminal propeptide domain of the $\alpha 1$ -chain of human type I and type III procollagen (Vuorio T, Mäkelä JK) were subcloned into the polylinker site of pGEM1 vector (Promega, Madison, WI). Before hybridization, the sections were deparaffinised in xylene and dehydrated in ethanol series. The sections were then treated with 0.2 M HCl for 20 minutes at room temperature (RT) and washed in DEPC- H_2O for 5 minutes after which they were treated with proteinase K (1mg/ml) for 30 minutes at 37 °C. They were then incubated in 0.2% glycine in PBS and washed twice in $1 \times \text{PBS}$ for 30 seconds. The sections were then postfixed with 4% paraformaldehyde in PBS for 20 minutes, washed in $1 \times \text{PBS}$ and acetylated in 0.25%-0.50% acetic anhydride in 0.1 M triethanolamine for 10 minutes, rinsed in $1 \times \text{PBS}$, dehydrated and air dried for 1–2 hours at RT. After this, the sections were treated with prehybridization mixture for 2 hours (10 mM DDT, 10 mM Tris-HCl, 10 mM NaPO_4 , 5mM EDTA, 0.3 M NaCl, 1 mg/ml Yeast tRNA, deionized formamide 50% and dextran sulphate 10% (w/v); 0.02% (w/v) Ficoll, 0.02% (w/v) polyvinylpyrrolidone and 0.02 mg/ml bovine serum albumin). They were then washed in $1 \times \text{PBS}$ and dehydrated. In the hybridization step, the probes were first denatured by boiling

them for 1 minute and placed on ice. 3×10^6 cpm of the ³⁵S-labeled antisense or sense probe in 40 μ l prehybridization buffer was applied on each section and the hybridization was carried out at 50 °C overnight. The posthybridization washes were performed as follows; twice at 50 °C for 1 hour in prehybridization mixture except for dextran sulphate and tRNA, rinsed for 15 minutes in 0.5 M NaCl in 10 mM Tris-HCl, 1mM EDTA (TE) at 37 °C, and then 30 minutes incubation in 0.5 M NaCl in TE containing 40 μ l/ml RNase A (Sigma, St Louis, MO) at 37 °C, washed in 15 minutes 0.5 M NaCl in TE at 37° C, 15 minutes twice in $2 \times$ standardized saline citrate (SSC) and 15 minutes twice in $1 \times$ SSC, both in 50 °C. The sections were dehydrated in graded series of ethanol containing 300 mM ammonium acetate and air dried at RT for 1 hour. The slides were then subjected to autoradiography by dipping them into NTB-2 film emulsion (Kodak) and were then put in light-tight boxes for 10–14 days. The slides were developed in D-19 developer (Kodak) fixed in Agefix (Kodak) and counterstained in hematoxylin-eosin.

4.10 Statistical analysis (III, IV)

Statistical analysis was performed using the SPSS software (SPSS Inc., Chicago, IL, U.S.A.). In paper III, Wilcoxon's signed-rank test was used to assess the statistical significance of the differences. The data are expressed as median and range (min-max). Kendall's tau-b correlation coefficient was used for the correlation analysis.

In paper IV, the independent samples or paired samples, when appropriate, T-test was used to assess the statistical significance of the differences. Linear regression was used for the correlation analysis. The data are expressed as means with standard deviations (SD).

5 Results

5.1 Purification and characterisation of the differently cross-linked carboxyterminal telopeptide structures (I, II)

The differently cross-linked and non-cross-linked carboxyterminal telopeptides were purified from human bone using preparative Sep-Pak C₁₈, S-100 HR size exclusion chromatography, C₈ and C₁₈ HPLC reverse phase runs under different conditions and finally a DEAE anion exchange HPLC column. The homogeneity of the purified antigens was tested by LDS-PAGE, which showed the peptides to migrate as one band, except for the monovalent form, which could not be visualised on the gel. The N-terminal sequencing revealed two known sequences for the trivalent ICTP peptide; TGDAGPV(GPU)₅ SAGFDFSFLPQPPQE corresponding to the α 1-C-telopeptide and GLUGTAGLUGM corresponding to the helical region of α 1-chain N-terminally from the cross-linking helical lysine residue 87. The same sequences were obtained for the divalent α 1_C α 1_H peptide, whereas for the divalent α 1_C α 2_H peptide the helical sequence obtained was GFUGTUGLUGF corresponding to the helical cross-linking site of α 2-chain next to the helical lysine 87. For the monovalent form, only one known sequence was obtained: TGDAGPV(GPU)₅SAGFDFSFLPQPPQE corresponding to the α 1-C-telopeptide.

For the divalent α 1_C α 1_H peptide, the MALDI-TOF mass spectrometry gave the following relative molecular masses: 5967.34, which would correspond to a peptide containing galactose moiety and one of the lysines being hydroxylated: 5983.86 with a similar peptide with one more lysine or proline being hydroxylated and 6128.93 which would contain glucosylgalactose and one of the lysines being hydroxylated (table 2).

In the MALDI-TOF mass spectrometry, the divalent α 1_C α 2_H peptide obtained relative molecular masses that would correspond to a peptide without the sugar moieties and without hydroxylation of the lysines (M_r 5858,52), a peptide with galactose (M_r 6036, 77) and a peptide with glycosylgalactose (M_r 6193,93) sugars attached with one of the lysines being hydroxylated.

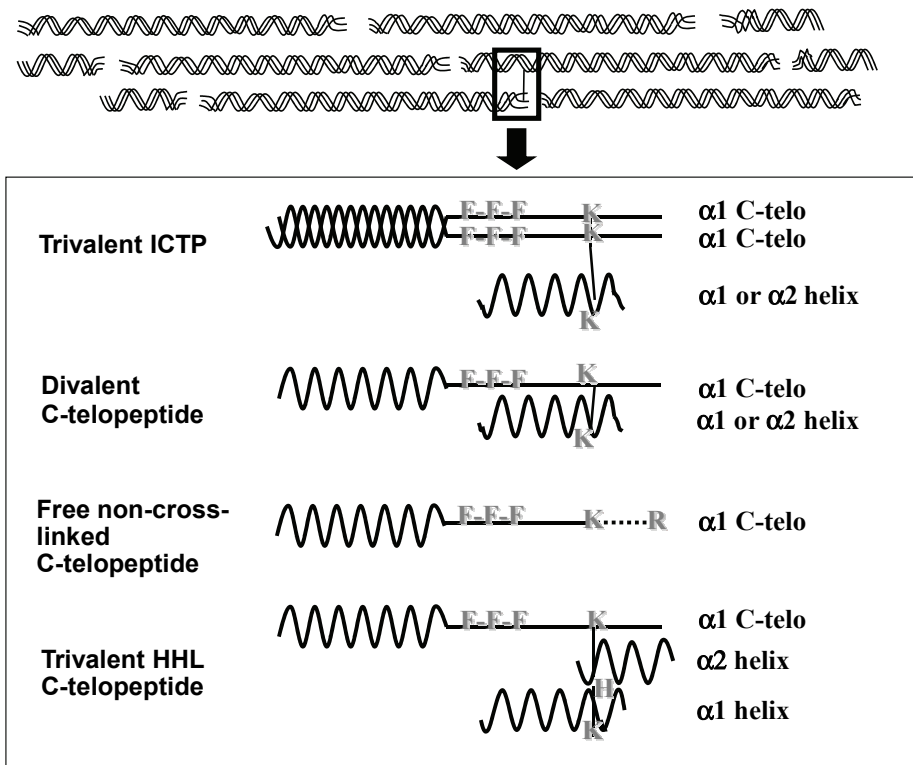


Fig. 4. A schematic presentation of the differently cross-linked and non-cross-linked carboxyterminal telopeptides. ICTP is the only structure containing two C-telopeptides cross-linked together and thus two phenylalanine rich regions (F-F-F). HHL = histidinoxylysinoxyloleucine, F = Phenylalanine, K = lysine or hydroxylysine involved in covalent cross-linking, H = Histidine, R = arginine. In the free non-cross-linked C-telopeptide the two possible cleavage sites of trypsin are marked with dotted line.

The purified monovalent $\alpha 1_C$ peptide did not have any sugars attached. There were two major forms detected in the MALDI-TOF mass spectrometry, the first cleaved after lysine and the other cleaved after arginine, six residues C-terminally from the (hydroxy)lysine. These peptides were present as differentially hydroxylated forms.

5.2 Cross-linking structures of the $\alpha 1(I)$ -C-telopeptides (I)

The $\alpha 1_C$ peptide did not contain any cross-links, further verifying it as the monovalent form. All cross-links present in the divalent peptides were previously characterised DHLNL and HLNL, the major cross-link in both peptides being DHLNL which requires that both cross-linking lysines must be hydroxylated. Slight differences were found in the relative proportion of HLNL cross-links. The $\alpha 1_C\alpha 2_H$ peptide contained relatively more of these cross-links compared to the $\alpha 1_C\alpha 1_H$ peptide, the ratio of DHLNL to HLNL being 17.5 in the $\alpha 1_C\alpha 1_H$ peptide and 3.4 in the $\alpha 1_C\alpha 2_H$ structure.

The overall content of pyridinium cross-links in the trivalent ICTP structure only was 0.42 residues/peptide. The majority of this was HP (0.36 res/peptide) with minor amounts of LP (0.06 res/peptide). About 60% of the cross-links in the ICTP structure were unknown of their character (see table 2 in paper I).

Table 2. The relative molecular masses of divalent and non-cross-linked carboxyterminal telopeptide structures obtained from MALDI-TOF mass spectrometry and theoretical masses of the peptides with different modifications, presuming that all prolines have similar hydroxylation level.

| Molecule | MALDI-TOF | | Theoretical M_r |
|---------------------------------|-----------|---|-------------------|
| Divalent $\alpha 1_C\alpha 1_H$ | 5967.34 | + Galactose + 1 Hyl + 1 Lys | 5964 |
| | 5983.86 | + Galactose + 2 Hyl | 5980 |
| | 6128.62 | + Glycosylgalactose + 1Hyl + 1 Lys | 6126 |
| Divalent $\alpha 1_C\alpha 2_H$ | 5858.52 | Without sugars + 2 Lys | 5854 |
| | 6036.77 | + Galactose + 1 Hyl + 1 Lys | 6032 |
| | 6193.93 | + Glycosylgalactose + 1Hyl + 1 Lys | 6194 |
| Free $\alpha 1_C$ | 3730.38 | Without sugars + Lys, cleaved after Lys | 3730 |
| | 4326.47 | Without sugars + Lys, cleaved after Lys | 4323 |

5.3 Immunoreactivity of the C-telopeptides in the ICTP and SP 4 immunoassays (I, II)

The SP 4 assay detected all the tested C-telopeptide antigens containing at least a single copy of the phenylalanine rich domain (figure 4). In the SP 4 assay, the strongest immunoreactivity was found towards the trivalent ICTP structure (Table 3). This is probably due to a collaborative effect of the two $\alpha 1$ C-terminal telopeptides, each containing one phenylalanine rich region, which together form the epitope of the ICTP assay. The synthetic peptide SP 4 and the purified free

$\alpha 1_C$ peptide showed about 1.3-fold better immunoreactivities than did the purified divalent $\alpha 1_C\alpha 1_H$. The $\alpha 1_C\alpha 2_H$ peptide exhibited a 2.85-fold difference to the SP 4 standard, which is approximately half of the immunoreactivity of the corresponding $\alpha 1_C\alpha 1_H$ divalent form. Possibly the sequence of the helical $\alpha 2$ chain can inhibit the immunoreaction by interacting with the epitopic phenylalanine rich domain, since it is more hydrophobic than the corresponding sequence in the $\alpha 1$ chain. The HHL cross-linked trivalent peptide had an immunoreactivity between those of the $\alpha 1_C\alpha 1_H$ and the $\alpha 1_C\alpha 2_H$. The ICTP assay was found to react only with the human ICTP antigen, which contains the phenylalanine rich regions of both C-telopeptides.

Table 3. Immunochemical comparison of differently cross-linked variants of the carboxyterminal telopeptide of type I collagen in the SP4 assay.

| Peptide | Slope | 50% intercept of inhibition (nmol/L) | -fold difference from SP 4 standard |
|---------------------------------|--------|---|--|
| SP 4 | -0.656 | 1.97 | 1 |
| monovalent $\alpha 1_C$ | -0.574 | 1.83 | 0.9 |
| divalent $\alpha 1_C\alpha 1_H$ | -0.562 | 2.50 | 1.3 |
| divalent $\alpha 1_C\alpha 2_H$ | -0.577 | 5.60 | 2.9 |
| trivalent ICTP | -0.575 | 0.34 | 0.2 |
| trivalent HHL* | -0.557 | 3.27 | 1.7 |

*histidinohydroxylysinonorleucine (HHL) cross-linked telopeptide ($\alpha 1_C\alpha 2_H\alpha 1_H$) isolated from human skin (Sassi *et al.* 2001).

5.4 The C-telopeptide structures of type I collagen in bone (I)

Human bone was found to contain about twice as much ICTP structure as previously reported for pyridinoline amounts in whole bone containing both the N- and C-terminal telopeptides (table 4). The cortical and trabecular bone did not seem to differ in their contents of the ICTP structure, although the number of subjects studied was very low. Although in cortical bone, the amount of ICTP seemed to decline over age, the result was not statistically significant possibly due to the low number of samples. The SP 4 to ICTP ratio indicated that there are other C-telopeptide structures present, such as the divalently cross-linked forms and non-cross-linked C-telopeptide.

Table 4. The concentrations of trivalently cross-linked ICTP peptide and the ratio of SP4 to ICTP in various human tissues solubilised by heat denaturation and trypsin digestion.

| Tissue | ICTP (mol/mol of collagen) | SP4/ICTP |
|-------------------------------|----------------------------|--------------------|
| Mineralised bone (n=5) | | |
| Cortical (CB) | 0.529* (0.014) | 1.70* (0.17) |
| Trabecular (TB) | 0.588*(0.011) | 1.62* (0.34) |
| Normal aortic valves | | |
| Age 18 (n=1) | 0.176 | 3.0 |
| Age under 50 years (n=10) | 0.077* (0.041) | 4.48* (1.13) |
| Age over 50 years (n=14) | 0.031* (0.011) | 5.40* (0.73) |
| Calcified aortic valves | | |
| non-mineralised matrix | 0.180* (0.034) | 1.73* (0.16) |
| calcified matrix | 0.183* (0.067) | 1.84* (0.28) |
| Achilles tendon (RUPT site) | | |
| normal, age 13 years (n=1) | 0.589 | 1.72 |
| normal, age 41–56 years (n=5) | 0.071* (0.017) | 6.29* (0.76) |
| ruptured (n=10) | 0.111** (0.059–0.148) | 4.11** (3.35–5.15) |

The values are expressed as means* with standard deviation in parenthesis, or median** with range in the parenthesis.

5.5 Type I and type III collagens in Achilles tendon (III)

The collagens accounted for 70% of total protein in Achilles tendon. No differences were found in the synthesis rates of either type I or type III collagen between the different sites, namely the rupture site (RUPT), control 1 (CONT1) or control 2 (CONT2), analysed by the immunoassays for the different propeptides (PINP, PICP and PIIINP).

In the insoluble matrix of the individuals with total Achilles tendon rupture, the amounts of both IIINTP and tryptic PIIINP were found to be markedly increased at the RUPT site compared to the control sites more proximally in the tendon (see table 1 in paper IV). A five-fold increase in the IIINTP content was seen also at the rupture site of the patients with total Achilles tendon rupture when compared to the same site in the cadaver samples. However, not such a clear increase was found in PIIINP levels, but there was a tendency in this direction.

The ICTP levels were surprisingly low in the tendon samples, being only 0.111 mol/mol of collagen at the rupture site (table 4). No differences were found RUPT and CONT1 sites, but the CONT2 contained slightly less ICTP than the other sites, probably due to the change of the tendon tissue to fascia. The ICTP

levels did not differ between the samples of the patients with total Achilles tendon rupture and the cadavers. The ICTP content declined during ageing at all other sites studied except for the rupture site of the samples of total Achilles tendon rupture. The ICTP content of one of the cadavers, a 13 years old male, was about 10-fold higher than in the other samples (0.589 mol/mol of collagen).

The SP4 to ICTP ratio again did not differ between the sites or between the rupture patients and cadavers, but there was a positive correlation between age and the SP4 to ICTP ratio at the CONT1 and CONT2 sites in both the rupture patients and cadavers, but only at the RUPT site in cadavers.

A semiquantitative reverse phase HPLC run was made from the RUPT site of one rupture patient and one cadaver. It clearly showed an increase in the IIINTP content at the rupture site of the patient with a total Achilles tendon rupture. The pyridinoline fluorescence was monitored during the run and a clear pyridinoline peak was shown at the site of IIINTP elution supporting the proposal that IIINTP structure contains pyridinium cross-links.

5.6 Type I collagen in human aortic valves (IV)

The type I collagen synthesis was increased in calcified stenotic aortic valves (AS) at both the mRNA and protein levels. The mRNA signals co-located with the calcified nodules in fibroblastic cells and also endothelial cells (see table 1 and figure 1 in paper III). Similarly, the propeptide levels of type I collagen were increased in the AS compared to the healthy controls, both PINP and PICP showing about 3-fold higher concentrations in the AS than either the healthy young group (YOUNG) or the healthy old group (OLD) (see table 2 in paper III). No change between the groups was observed in the PIIINP concentrations, although weak type III mRNA signals were observed in 13 of the 30 cases studied. The healthy control samples did not show any signals for either type I or type III collagen mRNA.

Despite the increase in synthesis in AS samples, the total collagen content was markedly reduced, comprising only 10% of total protein in the calcified matrix (CALC) and 30–40% in the matrix outside the mineral (SOFT). In the healthy control samples, collagen accounted for 90% of the total protein. The healthy samples had a relatively low ICTP content (YOUNG 77 mmol/mol of collagen, OLD 31 mmol/mol of collagen) for example when compared to that of bone (about 500 mmol/mol of collagen) (table 4). The AS samples had significantly increased ICTP contents with no difference between the calcified

and non-calcified matrix (SOFT 180 (SD 34) mmol/mol of collagen, CALC 183 (SD 67) mmol/mol of collagen) compared to the healthy controls. The ratio of SP4 to ICTP also changed, being in the healthy valves about 5 and in AS valves about 1.7 (table 4), pointing to a change in the cross-linked telopeptide structures. In the semiquantitative gel permeation run, the healthy control sample had a C-telopeptide structure larger than the ICTP structure, which was absent in the diseased valves. Both the SOFT and CALC fraction exhibited the presence of the divalently and non-cross-linked C-telopeptides (see figure 3 in paper III). The IIINTP content was significantly lower in the healthy OLD group compared to the other groups (YOUNG 0.40 (SD 0.06), OLD 0.25 (0.10), SOFT 0.37 (0.06) and CALC 0.38 (0.06) mol/mol of collagen).

The ICTP content decreased with age in the healthy samples ($r = -0.908$, $p < 0.001$) (see figure 4 in paper IV). At the same time the SP4 to ICTP ratio increased ($r = 0.538$, $p < 0.01$) being 4.48 (SD 1.13) in the YOUNG group and 5.40 (SD 0.73) in the OLD group. The IIINTP also displayed a decrease in concentration in conjunction with ageing ($r = -0.753$, $p < 0.001$).

The immunohistochemical staining revealed that the PINP and PICP were concentrated around the calcified nodules in the AS valves (see figure 5 in paper III). PIIINP staining appeared similarly to PINP and PICP. IIINTP staining was diffuse in the valve matrix. The ICPT staining in the calcified areas of diseased valves was moderate to strong and irregularly distributed.

6 Discussion

6.1 The C-terminal telopeptide structures of type I collagen in human bone

The differently cross-linked C-telopeptide structures were purified and analysed from human bone using an immunoassay specific for the trivalently cross-linked C-telopeptide structure (ICTP) and by developing an immunoassay based on the synthetic peptide of C-telopeptide of type I collagen (SP4) to allow monitoring of the peptides during the purification process. These assays were found useful to study the cross-linked type I collagen matrix in healthy human bone, healthy and calcified aortic valves and Achilles tendon samples from different sites of ruptured tendons. Traditionally, the collagen cross-linking has been studied using chemical cross-link analyses. However, these methods do not distinguish between the different collagen types or the location of the cross-link in either the N- or C-terminus. Cyanogen bromide or trypsin digestion and purification of these specifically cleaved peptides has also been used to characterise the differently cross-linked peptides from various tissues.

The epitope of ICTP assay was found to reside in the phenylalanine rich region N-terminally from the cross-linking site. The epitope comprises two $\alpha 1(I)$ telopeptides, which is found only in a structure commonly cross-linked with a pyridinoline or pyrrole type cross-links. It was found that the ICTP peptide purified from human bone contained only 42% of pyridinium cross-links (see paper I, table 2), with the nature of the remaining bonds being uncharacterised. The ICTP content in bone was approximately 0.53–0.59 mol/mol of collagen. If 42% of that is cross-linked with pyridinoline type cross-links, this would mean that the overall pyridinoline content was approximately 0.22 to 0.25 mol/mol of collagen. As such, this study is in agreement with earlier reports of the low pyridinoline content in bone (Eyre *et al.* 1988, Robins & Duncan 1987). Early explanations linked the low pyridinoline content of bone to the mineralization process. This was envisaged to prevent the conversion, or “maturation”, of the divalent cross-links to the trivalent forms, thus leaving a significant proportion of the cross-links in the divalent form (Eyre *et al.* 1988). Recently it was postulated that the lower levels of lysine hydroxylation within the telopeptide domains of bone collagen in comparison with cartilage was responsible for its lower pyridinoline content. Theoretically, this would favour the formation of trivalent

pyrrole-type cross-links but the majority (85%) of pyrrole-type cross-links are located in the N-terminus with only minor amounts at the C-terminus (Hanson & Eyre 1996).

The ICTP content measured in the mineralised compartments of healthy trabecular and cortical bone was approximately 2-fold greater than reported previously for the total pyridinoline content (accounting for both N- and C-terminal locations) in bone indicating again the presence of still uncharacterised cross-linking structures in ICTP. Further work is needed to establish the exact amount of pyrrole-type cross-links in ICTP, but it is unlikely that the pyrroles will account for the full trivalent cross-linking deficit of approximately 60%, currently identified in purified ICTP from human bone. Another possibility, i.e. the cross-linking structures would be formed through non-enzymatic advanced glycation end-products (AGE), is again unlikely since these linkages commonly form between helices. These kinds of compounds are unlikely to co-purify with ICTP fractions containing specific telopeptide-derived cross-links. AGEs are reported to increase in tissues with ageing (Saito & Marumo 2010), which is in contrast to the finding that there was a tendency to ICTP to decrease with age in healthy human bone and a clear decrease with age was found in healthy aortic valves and Achilles tendon.

ICTP has been found to be a good marker in many clinical situations. However, there has been some confusion regarding the usefulness of ICTP since it failed to reflect the response to estrogen or bisphosphonate therapy in osteoporotic patients. The epitope of ICTP assay was found to be destroyed by cathepsin K, the major collagenolytic enzyme present in osteoclasts. The C-telopeptide structure measurable with ICTP is produced by MMP-9 enzyme, the activity of which is often elevated in pathological conditions, such as metastatic prostatic or breast cancers, multiple myeloma and rheumatoid arthritis. Further evidence that the ICTP and Ctx represent different degradation pathways was obtained from the study of Garnero *et al* (2003), where MMP-2, -9, -13 and -14 released ICTP structure, but no detectable amounts of Ctx, whereas cathepsin K released large amounts of Ctx, but not ICTP. This feature of ICTP is clinically very useful, since it provides a reference to the clinician of the possible underlying pathological mechanism.

The SP4 assay was found to react with a structure consisting of at least one of these phenylalanine rich regions, thus reacting with all the purified C-telopeptide structures with different intensities. This property of SP4 undermines its use in assaying the contents of the different C-telopeptide structures directly from the

tissue samples without prior purification of the peptides. However, the SP4 assay is useful in following the peptides during different purification steps. When combined with ICTP, the ratio of SP4 to ICTP could be used as a marker of tissue “maturity” and a possible shift of the pathway in terms of collagen cross-linking. Any increase in the ratio of SP4 to ICTP would point to a relative increase in the amount of such variants of the cross-linked carboxyterminal telopeptide structures that can only be detected with the SP 4 assay. These include the free, non-cross-linked C-telopeptide, divalently cross-linked forms and the HHL-cross-linked C-telopeptide found predominantly in skin.

We found a considerable amount of free $\alpha 1(I)$ C-telopeptide in human bone. Previously it has been reported that bovine bone contains free $\alpha 1(I)$ chain. The authors presumed this was attributable to dissociation of divalent cross-links due to mineralisation (Otsubo *et al.* 1992). However, it seems unlikely that the mineralisation could weaken the collagenous matrix. Bailey speculated that it was an artefact produced by tissue processing, the EDTA decalcification destroying part of the divalent cross-links (Knott *et al.* 1997). However, in this study (I) the bone matrix was stabilised by reduction with NaBH_4 before the demineralisation process with EDTA. Other explanations for the free $\alpha 1(I)$ C-telopeptide could be the presence of newly synthesised collagen, which is not cross-linked but is embedded by mineral before cross-linking occurs. It has been shown that some malignancies are also characterised by the presence of free $\alpha 1(I)$ C-telopeptide structures (Kauppila *et al.* 1999) where the most likely explanation would be that the LOX activity is not able to keep in pace with the collagen synthesis. Another intriguing theory would be the presence of an $\alpha 1(I)$ homotrimer leaving some of the $\alpha 1(I)$ -chains free, if the third $\alpha 1$ -chain cannot find a cross-linking partner. The shorter C-telopeptide might be prerequisite for the correct alignment of the molecules to become cross-linked (Orgel *et al.* 2000). Homotrimeric type I collagen has been postulated to be present in osteoporotic and osteoarthrotic bones. The bone material used for the purification of the C-telopeptide structures in this study was obtained from patients undergoing hip replacement surgery, thus representing a mixture of both osteoporotic and osteoarthrotic bone.

6.2 Effects of ageing in the contents of the C-terminal telopeptide structures

It was found that the ICTP content decreased along with ageing in healthy aortic valves, normal Achilles tendon tissue and a tendency towards this decline was

found in a small number of normal bone tissue samples. For both Achilles tendon and aortic valve samples, there were also relatively young specimens (13 year old and 18 year old male, respectively) who had remarkably higher ICTP contents compared to the older samples. This finding is in agreement with other studies where the pyridinoline content has been reported to increase during the first decades and then to remain relatively constant through adult life in bone (Eyre *et al.* 1988, Oxlund *et al.* 1996).

In the healthy aortic valves, the ICTP concentration decreased with increasing age. In valves over 50 years of age, there was about half of the ICTP found in the younger group and 18-fold less ICTP than in normal bone. In the healthy aortic valves, the age related decrease in ICTP content and change in the SP4 to ICTP ratio is postulated to reflect a gradual shift in the cross-linking pathway. If the activity of the telopeptidyl LH isoenzyme were to decrease, this would result in a switch in cross-linking towards the Lys aldehyde pathway. Thus, in normal aging, the change could be, from pyridinoline toward a skin-like HHL or to an as yet uncharacterised cross-link.

6.3 Significance of enzymatic cross-linking of type I collagen in tissues

Collagen cross-linking has been shown to be of crucial importance for the formation and maintenance of functionally correct tissue (Oxlund *et al.* 1996). The clinical manifestations of rare genetic disorders like Bruck syndrome, characterised by fragile bones, joint contractures, osteoporosis, and short stature, and the kyphoscoliotic type of Ehlers-Danlos syndrome (type VI), with its characteristics of kyphoskoliosis, generalised joint laxity, skin fragility, and severe muscle hypotonia, are believed to be due to impaired collagen cross-linking. Many other more common clinical situations, such as many cancers, osteoporosis, osteoarthritis, rheumatoid arthritis and Paget's disease of bone, have as features increased turnover and abnormalities in the cross-linking of type I collagen.

The prerequisite for the cross-link formation is the conversion of the lysine and hydroxylysine residues to the corresponding aldehydes by the enzyme LOX. However, the cross-linking pathway is determined by the differential expression of the isoenzymes of LH in tissues. During recent years, it has become obvious that LH2 is the enzyme responsible for the hydroxylation of the telopeptide located lysines (van der Slot *et al.* 2003). Bruck syndrome, where defective

collagen cross-linking and low amount of pyridinoline is observed in bone but not in cartilage, is caused by mutations in the PLOD2 gene encoding for LH2 (Bank *et al.* 1999a). Overexpression of PLOD2 has been reported in some fibrotic situations, and has drastic effects in tissue like skin, where normally, it is the lysine aldehyde mediated cross-linking which prevails (Brinkmann *et al.* 2005, Van der Slot *et al.* 2003, Wu *et al.* 2006). Interestingly, in cell cultures of fibroblasts isolated from skin, treatment with β -aminopropionitrile (bAPN), an agent inhibiting cross-link formation by inhibiting LOX, did not affect the expression rate of PLOD2 in the cells (Wu *et al.* 2006). However, in cultures of MC3T3-E1 cells, which is a clonal preosteoblastic cell line derived from newborn mouse calvaria, both PLOD2 and LOX expression were downregulated by bAPN and homocysteine (Thaler *et al.* 2009). Another study indicated that osteoblastic gene expression is altered by the impaired cross-linking of the collagenous matrix. The mRNA expression of COL1A1 gene increased, when MC3T3-E1 cells were cultured in a matrix where collagen cross-linking was impaired by a former treatment by lathyrogenic substance, even though the substance itself had been removed from the matrix. (Turecek *et al.* 2008)

Evidence that a certain type of cross-linking profile is required for the regulation of mineralisation of tissues rather than being the consequence of mineralisation has been obtained from studies using canine healing callus (Wassen *et al.* 2000) and turkey leg tendon (Knott *et al.* 1997). In canine healing callus the mineralisation of the fibrils was normalised when the ratio of HP to LP level was that seen normally in bone. The importance of ECM in the formation of calcified structures is also evidenced by experiments showing that a matrix of appropriate composition and organization even in the absence of cells, can become calcified (Marsh *et al.* 1995).

6.4 Achilles tendon rupture

The ICTP content was surprisingly low at all the studied tendon sites. The human mineralised bone matrix contains 3–6 fold more ICTP structures than Achilles tendon matrix. The ICTP content decreased and the ratio of SP4 to ICTP increased with age, indicative of a relative increase in the amount of such variants of the cross-linked carboxyterminal telopeptide structures that can only be detected with the SP 4 assay. It is unlikely that these would be the immature, divalently cross-linked structures, since one would not expect advancing age to increase the number of divalent structures, which are precursors of the trivalent

forms. This telopeptide structure appearing into the Achilles tendon over age could be identical or analogous to the structure predominating in human skin, which is cross-linked by the histidinohydroxylysinonorleucine (HHL) cross-link. It has been previously shown that a reduction of the pyridinoline cross-link density causes biomechanical weakening of healing rabbit medial collateral ligament. The authors discussed the possibility that skin-like cross-linking might be the reason for the decrease in the pyridinoline content (Frank *et al.* 1995). A change in the ratio of SP4 to ICTP is compatible with this suggestion, since the HHL-cross-linked carboxyterminal telopeptide structure can be measured by the SP4 assay, but not with the ICTP assay. If the low ICTP content contributes to total Achilles tendon rupture, it would appear that the change in the collagen fibril organization is a generalized phenomenon within tendon tissue rather than a local disturbance.

The content of cross-linked type III collagen was markedly increased in the insoluble tissue digests of the rupture site, which contained 5.1-fold and 12.5-fold amounts of IIINTP compared to the control 1 and 2 sites, respectively. IIINTP represents type III collagen which has been incorporated into the collagen fibrils and stabilised there by intermolecular cross-links. The PIIINP levels in the soluble tissue extracts showed no differences between the sites, pointing to a slow overall synthesis rate of type III procollagen at all sites of the tendon. Thus, the accumulation of such a large amount of IIINTP at the rupture site must have taken place over a longer period of time. This suggests that there has been a continuous, long-lasting microtraumatic process prior to the total rupture of the Achilles tendon. The cause of the microtraumatic process taking place in the Achilles tendon prior to the rupture remains obscure.

6.5 Aortic valve calcification

The collagen cross-linking in aortic valves has not been extensively studied. However, as the healthy valve is a thin, pliable tissue, subjected to extreme extensile strength demands, it is not likely that it would normally be cross-linked, at least completely by the cross-links derived from the hydroxylysine aldehyde pathway. Interestingly Aldous and colleagues (2009) found the major collagen cross-link in (young) adult bovine aortic valve to be HP, with minor amounts of HHL. Again, Balguid *et al.* (2007) reported that healthy middle aged human aortic valve contained 0.26 ± 0.05 mol/triple helix of HP. These workers did not measure other cross-linking structures (Balguid *et al.* 2007). In this present study,

the healthy human aortic valves contained only 0.077 and 0.031 mol/mol of collagen of ICTP, far less than these two other studies reported for the pyridinoline content. However, ICTP detects only the trivalently cross-linked structures of the C-terminus of type I collagen. The remainder of the pyridinoline type cross-links may reside in the N-terminal telopeptide of type I collagen or in other collagen types. A healthy valve structure contains also a significant amount of type III collagen, which is also cross-linked by the pyridinoline type cross-links. Further work is needed to establish the cross-linking profile of aortic valves in health and disease.

With calcification the ICTP level increased in aortic valves, though it still remained less than in healthy human bone. The ratio of SP4 to ICTP was higher in the normal valves than in their calcified counterparts, indicative of the relatively higher presence of cross-linked structures other than trivalent ICTP in healthy valves. In calcified valves, the SP4 to ICTP ratio decreased to that observed in bone. A change in the cross-linking profile in the AS valves was also observed in the size exclusion chromatography analysis. The appearance of divalent and non-cross-linked C-telopeptide structures in AS valves may be related to the presence of mineral, which would prevent the maturation of the immature divalent cross-links to their trivalent forms, as has been postulated to occur in bone. However, as the matrix surrounding the mineral in AS showed a similar cross-linking profile to the mineralised matrix, an alternative explanation could be that there is active remodelling and thus predestination of the matrix to become mineralised, as suggested also to occur in mineralising turkey leg tendon tissue (Knott *et al.* 1997). The increased turnover in the AS matrix may contribute to the appearance of the immature C-telopeptide structures, as they may represent the newly laid matrix, where the cross-links have not had time to mature into the trivalent structures.

The mechanism for the ICTP increase in AS valves may be some kind of selective degradation of the other cross-linked and non-cross-linked C-terminal telopeptide forms, since these may be more susceptible to enzymatic degradation, leaving a collagenous matrix enriched in ICTP-like structures. Another possible explanation for ICTP elevation is that there is an active shift towards the formation of trivalently cross-linked structures detectable with the ICTP assay through changes in the post-translational modifications of collagen, such as lysyl hydroxylation of the telopeptides and pyridinoline-type cross-link formation. Nonetheless, the increased content of trivalently cross-linked ICTP most probably contributes to the increased stiffness of the valve because of its more stable

properties and its increased resistance to degrading enzymes. It can also indirectly influence or be a marker of molecular organization of collagen in the fibrils that is more favourable for the accommodation of mineral.

In organ cultures of normal, rheumatic and floppy human heart valves, it has been observed that collagen synthesis was increased in abnormal heart valves. This was accompanied by increase in collagen and total protein levels (Henney *et al.* 1982). Elevated collagen synthesis has been reported in mitral valves subjected to altered stress during mitral regurgitation and abnormal ventricular wall motion in sheep (Quick *et al.* 1997). The same authors reported later that despite the increased collagen synthesis, the total collagen content was decreased in these valves. They considered this decline to be due to a possible decrease in type III collagen production (Kunzelman *et al.* 1998). In this study the collagen content was clearly decreased in diseased valves although type I collagen synthesis was increased and type III collagen synthesis remained the same. The increased turnover seemed to be located particularly around the calcified nodules. Transforming growth factor- β 1 (TGF- β 1) is thought to be a major contributor in atherosclerotic calcification, its effects being mediated by its ability to increase the production of type I collagen and fibronectin. However, recently some authors have claimed that an increase in the activity in certain matrix metalloproteinases (MMP) in AS is either due to increased transcription of the enzymes or to an imbalance between the expressions of MMP and their inhibitors (Edep *et al.* 2000, Fondard *et al.* 2005). A reduced collagen content has been reported to be associated with the increased stiffness of ageing rat aorta and it may also contribute to the impaired function of the valve in AS (Brüel & Oxlund 1996).

7 Conclusions

1. About 60% of ICTP is cross-linked with a presently uncharacterised cross-linking structure, as the pyridinium cross-link concentration accounted for only 0.42 residues per ICTP peptide. All of the divalent C-terminal telopeptides are cross-linked with known DHLNL and HLNL cross-links. A non-cross-linked C-telopeptide structure was found to be present in bone.
2. The epitope of the ICTP assay resides in the phenylalanine rich region present in the C-terminal telopeptide of $\alpha 1$ chain of type I collagen. Two of these regions need to be present in the antigen before it can be recognised in the ICTP assay.
3. The ICTP antigenicity is lost with cathepsin K treatment, but not with MMP activity. Thus, the ICTP assay reflects the MMP mediated degradation, a condition present in many pathological situations such as rheumatoid arthritis, breast or prostate cancer with bone metastases, multiple myeloma.
4. The ruptured Achilles tendons contain significantly more type III collagen than their healthy counterparts. The accumulation of type III collagen is a slow process and a local phenomenon at the rupture site, pointing to a long-lasting continuous microtraumatic process before the total rupture of the Achilles tendon. The low level of ICTP structures in the tendon tissue may additionally affect the quality of the matrix. These findings clearly point to the presence of qualitative changes which decrease the strength of the matrix even before the total rupture of Achilles tendon actually occurs.
5. In calcified stenotic aortic valves the ICTP content was increased and the ratio of SP4 to ICTP decreased indicative of a change in cross-linking towards the hydroxylysyl pathway. The total collagen content was dramatically decreased in the calcified valves.

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