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Biochemical and mechanical properties of subchondral bone in osteoarthritis

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Abstract. The subchondral bone has long been known to thicken in osteoarthritis. However, recent evidence has demonstrated that the turnover of the bone is increased several fold, and further suggests that the thickening occurs prior to degradation of the articular cartilage, indicating that it plays a role in the pathogenesis of osteoarthritis. The mechanical and biochemical properties of the subchondral bone are therefore of particular interest in any attempt to determine the nature of the factors initiating osteoarthritis.

We have shown that the subchondral bone collagen of the femoral head possessed a 20-fold increase in turnover, as assessed by procollagen rate of synthesis and metalloproteinase degradation, and a 25% decrease in mineralisation. This increased metabolism and high lysyl hydroxylation leads to narrower and weaker fibres. Additionally the phenotypic expression of the osteoblasts is modified to produce increasing proportions of type I homotrimer in addition to the normal type I heterotrimer, which further reduces the mechanical strength of the bone. Overall, the narrow immature collagen fibres, the reduction in pyrrole cross-linking, decreased mineralisation, and increased amounts of type I homotrimer, all contribute to a weakening of the mechanical properties of the subchondral bone.

Keywords: Subchondral bone, osteoarthritis, collagen, cross-linking, metabolism, biomechanics

The majority of the basic research into the pathogenesis of osteoarthritis (OA) has concentrated on the mechanisms involved in the characteristic focal destruction of the articular cartilage. The initiating events of this destruction are believed to be changes in the proteoglycans followed by the collagenous framework, where after the disease is irreversible.

The well recognised additional characteristics of bony osteophytes and subchondral bone thickening have to a large extent been considered secondary and unimportant in the pathogenesis of osteoarthritis. However, some workers have suggested the involvement of bone. Over four decades ago Johnson [29] postulated that subtle changes in bone remodelling might precipitate irregularities in the articular cartilage. Radin and Rose [48] later proposed that the increased bone mass and sclerosis of the subchondral bone plate would cause stiffening of the bone and result in cartilage destruction on repetitive loading. More recent studies have re-emphasised the importance of the bone changes. Newer radiographic techniques [11] have demonstrated progressive thickening of the subchondral bone with joint narrowing and technetium-labelled bisphosphonate demonstrated that the heightened bone activity of the knee was related to the progression to severe OA, as monitored radiographically by joint space narrowing [20].

The question then arises as to whether the bone thickening was a result of cartilage degradation, occurred at the same time, or preceded cartilage degradation [4,12,52]. If the latter, then the subchondral

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bone thickening is indeed important in the pathogenesis of OA, either as an initiating factor or, at the very least, important in the progression of OA. To follow the progression of OA recourse has to be made to animal models. Thickening of the subchondral bone was shown to occur prior to cartilage destruction in elderly cynomolgus macaques and that the thickness of the subchondral bone was related to the onset of cartilage fibrillation [14]. We have demonstrated a similar finding during spontaneous OA in the guinea pig knee [3] and have reported that remodelling of the cruciate ligament also occurred prior to cartilage destruction [2,47]. The latter finding may be related to the changes in the subchondral bone because of a change in stress on the bone due to the remodelling of the ligament. In a mechanically induced model it was reported that sub-fracture impacts applied to the patellofemoral joint of rabbits resulted in subchondral bone thickening after 6 months whilst cartilage stiffness only decreased after 12 months [43]. In an attempt to detect earlier changes than the histological techniques revealed ultrasonic echography was employed [51] in a rodent inflammatory joint model and reported changes in both bone and cartilage before any histological evidence of cartilage damage.

However, regardless of whether thickening of the subchondral bone occurs at a late stage or precedes cartilage destruction it is important to know the biochemical and mechanical properties of bone, since they may be important in the pathogenesis of OA. In an analysis of human femoral head bone collagen we found that the collagen was turning over at a rate several fold greater than non-OA bone [39] and is not a slow fibrosis due to remodelling of micro-fractures as suggested by Radin [48]. The increased rate of synthesis (20-fold) was determined from the levels of the C-terminal pro-peptide of type I collagen (PICP) and the increased degradation by the levels of MMPs (20-fold) (Fig. 1). The elevated type I collagen synthesis was accurately corroborated by the increase in alkaline phosphatase activity. The relative contribution of cathepsin K, which has been shown to cleave the collagen triple helix at several locations [23] and the MMPs remains to be determined. Transforming growth factor-beta (TGF-β) levels were increased 4-fold. TGF-β plays a major role in remodelling and recruitment of osteoblast precursors [46], stimulating collagen synthesis, inhibiting MMPs and increasing TIMPs thus leading to a net gain in collagen deposition. In contrast the mineral content of the new bone was significantly reduced (30%) (Fig. 2) which is consistent with previous studies reporting hypomineralisation of the

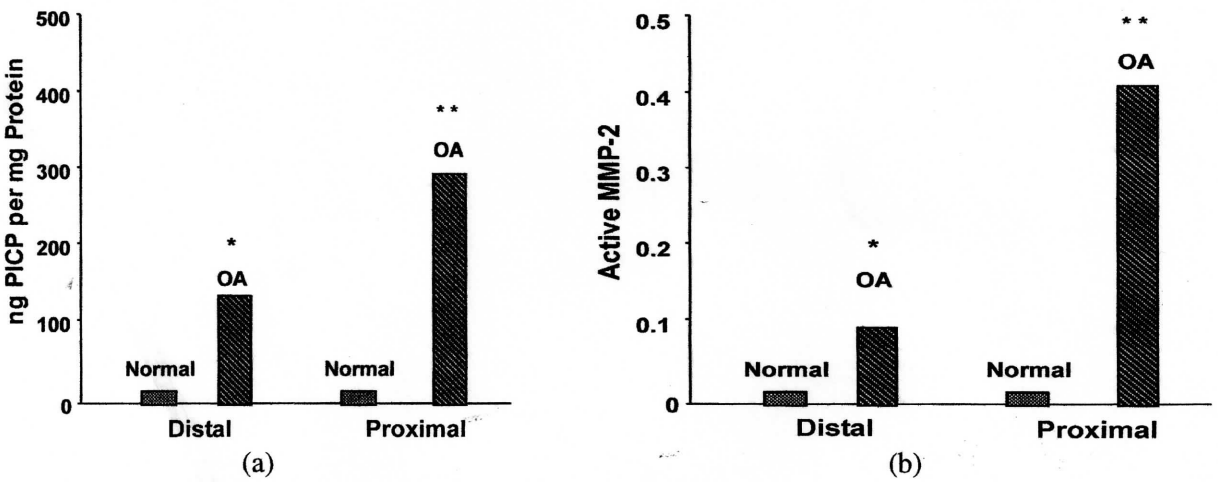

Fig. 1. Comparison of the metabolism of subchondral and normal bone from the femoral head; (a) relative rate of synthesis of type I collagen determined by type I propeptide (PICP) antibody in both distal and proximal regions of the femoral head. (b) Relative levels of MMP-2 determined by zymography against a standard MMP-2.

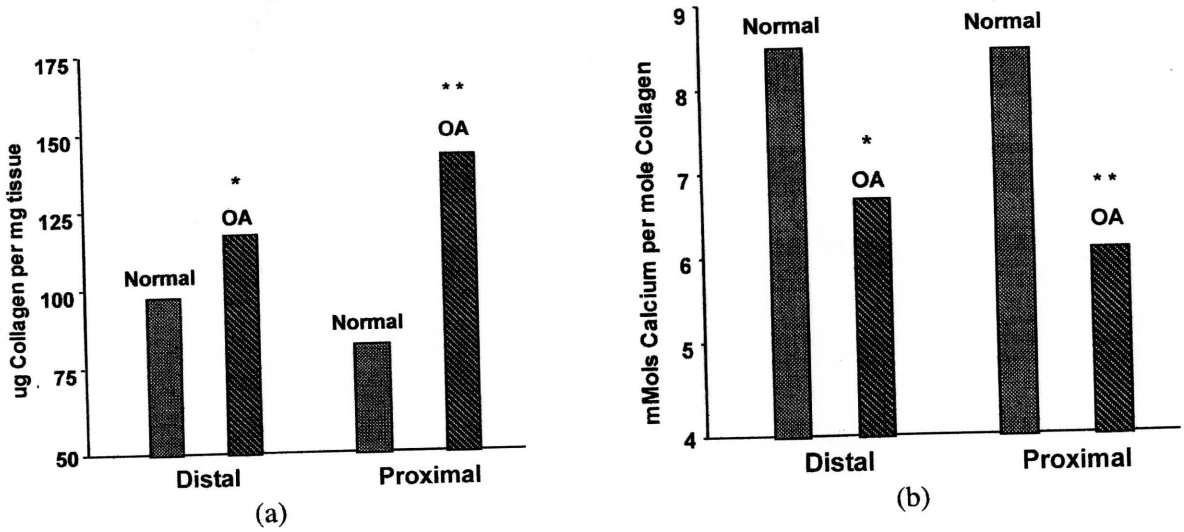

Fig. 2. Comparison of the quality of the subchondral bone (a) collagen content as μg per mg of bone (b) mineralisation in terms of mM calcium per mole of collagen, in distal and proximal domains of the femoral head. ($^{*}p < 0.005$ and $^{**}p < 0.001$.)

bone [25] and the increased presence of osteoid [50]. The greatest increase in the rate of turnover was in the subchondral bone plate but high levels were maintained in the distal regions of the femoral head. The higher turnover rate would lead to a higher proportion of immature bone suggesting a reduced mechanical strength. Indeed, a detailed biomechanical analysis reported that cancellous bone from OA femoral head was significantly weaker than age-matched controls [36].

The increased metabolism of bone collagen may be a feature of other bones in OA subjects. Interestingly, increased bone density and increased levels of TGF-β have been reported in the iliac crest bone of OA subjects [18] and they suggest that the increased bone density of OA subjects protects them against osteoporosis [19]. Certainly spontaneous fracture of femoral heads tends not to occur in OA subjects.

Of additional importance is the fact that the increased turnover is not a function of age. We have investigated bone collagen turnover in the iliac crest bone collagen over a wide age range of mature non-OA subjects. These individuals did not show any changes in rate of turnover or the nature of the stabilising cross-links through into old age. There was, as expected, a steady loss of bone collagen, which correlated with the loss of density and mechanical strength [5]. Any changes found with increasing age are, therefore, likely to be due to disease processes, for example, in the case of osteoporosis the increased hydroxylation leads to narrow fibres, increased pyridinoline cross-links and loss of strength [40].

Simplistically bone can be considered to be a two phase system of a collagenous framework providing tensile strength and hydroxyapatite providing rigidity to that framework. The collagen fibres themselves are formed by a quarter-staggered end-overlap parallel alignment of the collagen molecules. This very precise organization is crucial to both the stabilisation of bone by intermolecular cross-linking and the nucleation of mineralisation within the gap region of the fibre. The crystals of apatite are initially formed in the gap region and have been shown to be plate-like crystals penetrating the fibre prior to covering the fibre surface [34]. The remodelling of bone therefore requires several stages, removal of the mineral, degradation of the collagen, re-synthesis of the collagen and finally re-mineralisation.

The tensile strength of the collagen framework is provided by the intermolecular cross-linking of the collagen molecules making up the fibres of the framework. The initial head to tail cross-linking occurs enzymically through lysyl or hydroxylysyl aldehydes formed in the non-helical telopeptide region by the

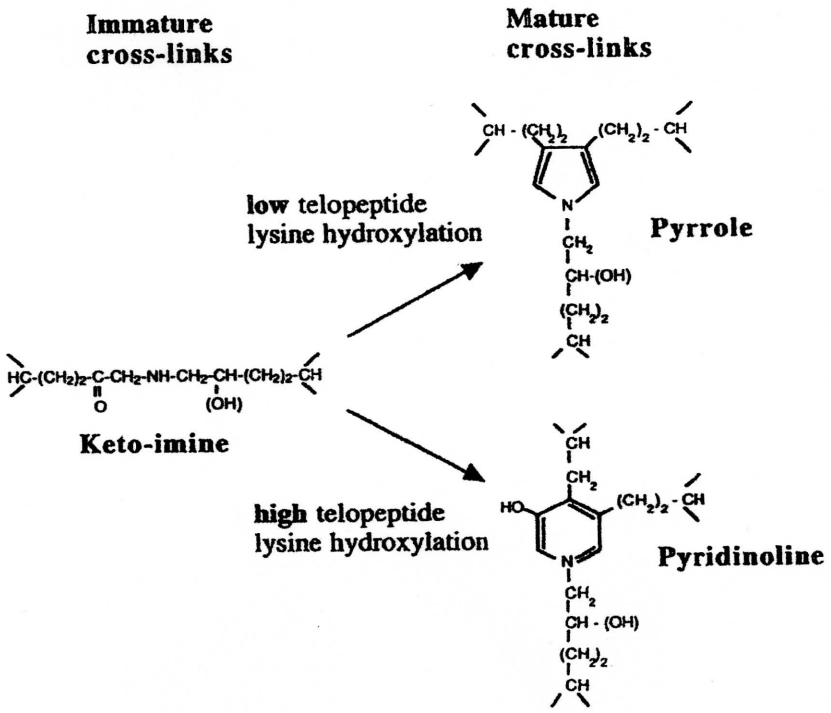

Fig. 3. Alternate pathways for the maturation of the divalent keto-imine to the mature trivalent hydroxylysyl and lysyl-pyrrole or pyridinoline cross-links.

action of lysyl oxidase. In the case of bone collagen these lysyl aldehydes then react with the ε-amino group of specific lysine residues within the triple helical domain exactly opposite the telopeptide to form the keto-imine intermolecular cross-link. These divalent cross-links are capable of providing tensile strength to young bone collagen but spontaneously react with another lysyl or hydroxylysyl aldehyde to form a trivalent mature cross-link, the pyrroles and the pyridinolines respectively (Fig. 3). The proportion of these mature cross-links increases as turnover decreases with maturity. The relative amount of each particular cross-link depends on the relative levels of hydroxylation of the triple helical and telopeptide lysines, each site being controlled by a different lysyl hydroxylase [7]. In addition there is no oxidizable lysine in the C-telopeptide of the α2(I) chain thus restricting the type of cross-link at the C-terminal. The pyrrole cross-link appears to be concentrated at the N-terminal whereas the pyridinolines are present at both termini [26], although there is more lysyl pyridinoline than hydroxylysyl-pyridinoline at the N-terminus. This finding suggests a distinct role for each cross-link and in view of the apparent correlation of the pyrroles with bone strength we have proposed that the latter form interfibrillar cross-links [31]. The hydroxylation state of the telopeptide lysine residues is clearly crucial to the type of immature and subsequent mature cross-link, and hydroxylation of the specific triple helix lysine determines whether it is the lysyl or hydroxylysyl derivative of the cross-link.

The bone matrix varies with skeletal site reflecting the different functions of bone. For example, a study of avian bone [32] revealed a higher turnover of the collagen in the tibiotarsus and increased lysyl hydroxylation compared with the humerus. Variable turnover rates must also occur in different human bones and will also influence the lysine hydroxylation and the nature of the cross-links and therefore their properties. This suggestion is currently being investigated.

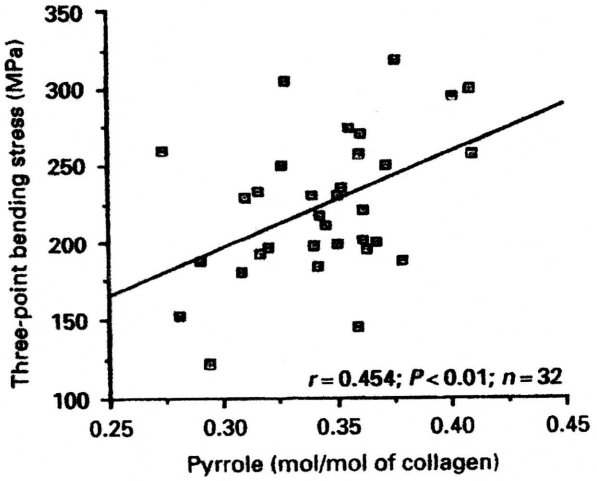

Fig. 4. Correlation of the pyrrole cross-links and the strength of bone.

A major clinical problem in bone biology is the prediction of bone strength. There is no doubt that the amount of bone per unit volume (BV/TV), or apparent density, is a major parameter influencing the stiffness and strength of bone but a substantial part of the variance of these mechanical properties, as much as 30%, is unexplained [33,42]. Several studies have suggested that the unexplained variation is due to the different trabecular architecture, for example, the thickness, spacing and connectivity of the trabeculae [15], but this still does not permit a total prediction of the properties [17]. However, despite its role as the fundamental framework of bone few attempts have been made to include the nature of the collagen cross-links in the prediction of the mechanical properties of bone. As discussed above the stability of the collagen may vary depending on the nature and extent of the collagen cross-links. It has long been known that inhibition of the lysyl oxidase results in bone of little or no tensile strength. Even a partial reduction in the extent of cross-linking produces a significant loss of mechanical strength in the bone [30,35,44].

Initial attempts to correlate mechanical strength and intermolecular cross-linking revealed a surprising relationship between the tensile strength and the pyrrole cross-link rather than the pyridinoline cross-link (Fig. 4). These studies were carried out on avian tibiotarsus bone employing three point bending to determine the tensile strength [30]. Further studies on avian bone, during an assessment of genetic selection for resistance to osteoporosis, again revealed a positive correlation between humerus and tibiotarsal pyrrole content and bone strength [54].

Encouraged by these relationships we carried out a more detailed study of the mechanical properties of human vertebral cancellous bone and the nature of the intermolecular cross-linking of the collagen. We determined the density with peripheral quantitative computer tomography (pQCT) and mechanically tested the bone by compression [8,9]. The amount of collagen and the nature of the cross-links were determined on these samples and on applying multiple regression we found that the prediction of mechanical properties was improved by combining the cross-link assessment with density.

Further, the hydroxylysyl-pyridinoline/lysyl-pyridinoline ratio appeared to be a significant predictor of strength ($p = 0.001$) and stiffness ($p = 0.001$), samples with a high ratio being stronger and stiffer. The ultimate strain correlated with the hydroxylysyl-pyridinoline or lysyl-pyridinoline concentration. However, there was a wide variation between the different subjects analysed. This variation is caused

by the different degrees of lysyl hydroxylation, which is controlled by different isomers of lysyl hydroxylases acting on either the specific lysines in the triple helix or in the non-helical telopeptide. These variations lead to different cross-links. The variation of hydroxylysyl- and lysyl-pyridinolines between subjects indicates that the activity of the lysyl hydroxylase acting on the triple helical lysines also varies significantly from one subject to another.

In a continuation of these studies on the vertebral cancellous bone we compared the nature of the collagen with the structural organisation of the trabecular network [9]. This bone has a low bone volume fraction (BV/YV); only 7–14% of the total bone is occupied by bone tissue, the rest being marrow. We determined bone volume fraction, BV/TV; trabecular thickness, TbTh; trabecular number, TbN; trabecular spacing, TbSp; strut length TSL; number of nodes, Nd; number of free ends, Fe; and amount of osteoid bone. Again we observed a relationship between the nature of the bone matrix and the concentration of the cross-links (the pyrrole and pyridinolines) and the structural organisation of the trabeculae. The concentration of pyrrole and pyridinoline cross-links correlated with the structural organisation, but in opposite directions. Thus, the pyrrole/pyridinoline ratio was a good predictor of the above parameters. Subjects with high pyrrole and low pyridinoline in their bone collagen had a thicker and simpler structure, whereas those with a low pyrrole and a high pyridinoline content possessed thin trabeculae that were more numerous. This indicates a relationship between the structure and the stability of the collagen fibre controlling the micro-architecture of the bone. Again high pyrrole appears to be important and this indicates that the regulation may be in the rate of collagen turnover and the activity of the lysyl hydroxylase.

We have not, as yet, carried out a similar study of the cancellous subchondral bone of the OA femoral head. However, based on the above studies on the vertebral bone the high pyridinoline low pyrrole ratio observed in OA subchondral bone should result in weaker and narrow numerous struts, which is consistent with our findings to date. Indeed, morphological changes, which are different from those associated with ageing, increased trabeculae number and reduced separation between trabeculae have been reported for OA bone [22].

The extremely rapid turnover of the collagen in OA subchondral bone means that the environment of the osteoblasts is very different from normal bone, and the consequent change in cytokines and growth factors could lead to differences in their phenotypic expression. Indeed, osteoblasts isolated from the bone of OA subjects were found to be capable of degrading cartilage proteoglycans in contrast to osteoblasts from normal bone [55] and possessed an altered response of OA osteoblasts to various cytokines and growth factors [27]. We therefore investigated the phenotypic expression of the osteoblasts *in situ* and demonstrated the presence of collagen, type I homotrimer $[\alpha 1(I)]_3$, in OA femoral heads in addition to the normal type I heterotrimer $[(\alpha 1)_2\alpha 2]$. The amount of the homotrimer, demonstrated by the ratio of the $\alpha 1$ to $\alpha 2$ chains varied considerably from subject to subject, from 4 : 1 to 17 : 1 compared with 2 : 1 for normal bone [6] (Fig. 5).

The amount of the excess $\alpha 1$ chain presumably depends on the rate of turnover and hence the severity of the OA, but further studies are required on clinically classified samples to confirm this suggestion. Type I homotrimer has previously been reported in tumours and in embryonic tissue, again tissues similarly involved in a high turnover of collagen, but were reported to be genetically distinct collagens. However, cyanogen bromide cleavage of the homotrimer derived from OA subchondral bone revealed an identical peptide profile to the $\alpha 1$ chain of the heterotrimer isolated from the type I collagen of non-OA bone [6]. The presence of the homotrimer is therefore due to a loss of the regulation controlling the $\alpha 1/\alpha 2$ ratio to produce an excess of $\alpha 1$, rather than a genetically distinct type I collagen $\alpha 1$-chain.

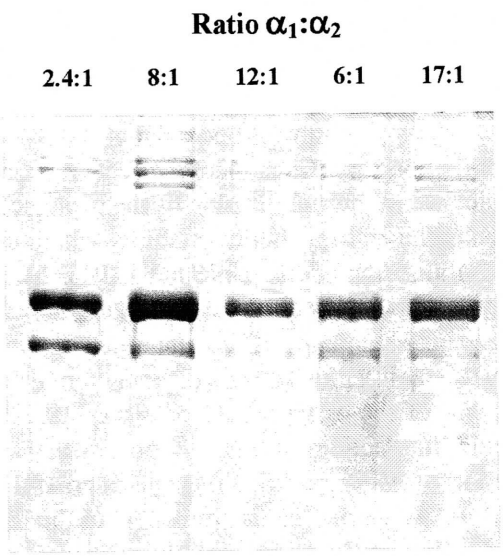

Fig. 5. SDS gel electrophoretogram demonstration of increasing ratio of type I $\alpha 1/\alpha 2$ from 2.4 : 1 for normal bone ranging up to 17 : 1 for the subchondral bone, indicating the presence additional $\alpha 1$(I) chains of type I homotrimer.

The identification of type I homotrimer prompted us to search for a possible association of Col1A1Sp1 polymorphism in OA [37] by employing a modification of the technique for a Col1A1 association in osteoporosis [24] we observed moderate evidence for increased type I homotrimer in OA females. Although not statistically significant the result was consistent with increased synthesis of $\alpha 1$(I) collagen and hence the presence of the homotrimer.

To determine the effect of increasing amounts of type I homotrimer on the mechanical properties of the OA subchondral bone we studied the mouse model expressing a variant of osteogenesis imperfecta (*oim*) in which the $\alpha 2$ chain had been deleted [15]. This *oim* mutation is a single nucleotide deletion in the ColA2 gene that alters the terminal 50 amino acid residues of the pro-$\alpha 2$(I) chain and prevents its association with the pro-$\alpha 1$(I) chains to form the type I heterotrimer. The mechanical strength of the bones of these animals is about half that of the wild type mouse [13]. X-ray diffraction studies of the tail tendon indicated an altered collagen structure [38], but the striated pattern of the fibre in the electron microscope appeared normal suggesting that the alteration is small. However, the covalent intermolecular cross-links are the major factor in governing the mechanical strength of the fibre and we have demonstrated a significant decrease in the cross-linking compared with the wild type mouse, which would certainly account for the loss of mechanical strength. Quite why the loss of the $\alpha 2$ chain should have such an effect on the cross-linking is not immediately obvious, particularly since the deletion did not appear to affect the molecular alignment of the molecules in the fibre as observed in the electron microscope. In addition the mutation would certainly not have affected the activity of the lysyl oxidase. To account for the decrease in cross-linking we carried out a detailed study of the integrity of the triple helix of the homotrimer by differential scanning calorimetry. The denaturation temperature (Td) of the *oim* molecule was 2.6°C higher than the wild type and the Tm for the *oim* fibre was 1.4°C higher than the wild type fibre. Previous studies had reported identical denaturation temperatures using the less accurate circular dichroism [38]. The increase in denaturation temperature is presumably due to the increase in hydroxyproline content on replacement of the $\alpha 2$ chain by the $\alpha 1$ which contains a higher hydroxyproline content, with 348 hydroxyprolines compared with 327 for the heterotrimer triple helix.

The mechanism of stabilisation of the triple helix by hydroxyproline is still controversial. It may form stabilising hydrogen-bonded water-bridges as originally proposed by Ramachandran [49] a proposal currently supported by recent X-ray studies defining the location of the water molecules on the triple helix [10]. Alternatively other workers [28] have proposed that the hydroxyproline stabilises the *trans* configuration of the prolyl peptide bond by the inductive effect of its hydroxyl group suggesting that water hydrogen bonding plays no role in the stabilisation of the triple helix. This is supported by earlier work on the effects of alcohol on collagen triple helical stability which demonstrated that water was not an essential component in the stabilisation of the triple helix [21]. In contrast, our recent NMR/DSC studies indicate that H-bonded water does play a role in stabilizing the collagen molecule [53].

We found only a small decrease in the enthalpy of the *oim* fibre suggesting that the amount of disorder of the triple helical molecules was small. However, the difference in denaturation temperature between the molecules in solution and that of the aggregated fibres was 19.9°C against 23.1°C for the wild type, indicating reduced molecular interactions and hence looser packing of the molecules in the fibre. Calculation of the volume fraction of water revealed that the interaxial separation of the molecules in the *oim* fibre was increased by 1.4 Angstrom. This is equivalent to the length of a C–C bond and would therefore certainly decrease the ability of the telopeptide lysine-aldehyde to interact with the ε-amino group from an adjacent molecule to form an intermolecular cross-link. We therefore proposed that the decrease in cross-linking and hence in mechanical properties, was due to the increased water content of the fibre rather than a distortion of the molecular structure. An increasing proportion of the weaker type I homotrimer fibres in the subchondral bone of OA subjects would clearly have a significant effect on decreasing its mechanical properties.

The rapid turnover and fibrosis of bone collagen together with the ability of the 'modified' osteoblasts to degrade articular cartilage [55] need to be taken into account by tissue engineers attempting to replace, or regenerate, the articular cartilage of osteoarthritic femoral head and knee bones.

It is clear that osteoarthritis is a disease encompassing articular cartilage degradation and fibrosis of the subchondral bone and therapies need to consider both aspects of the disease rather than concentrating solely on the articular cartilage. Partial regression of the symptoms of OA of the knee have been achieved by high tibial osteotomy [1] which reduced the subchondral thickening. More recent studies [45] have proposed an application of licofelone to control abnormal bone remodelling in OA since licofelone was able to inhibit OA osteoblast prostaglandin E2 and leukotriene B4 *in vitro*.

In summary, the ability of bone collagen to provide a strong framework and to fully mineralise depends on the very precise alignment of the type I collagen heterotrimer. However, in osteoarthritis the increased narrowing of the fibres diameters, the reduced level of the pyrrole cross-link, the decreased mineralization, together with the increasing proportion of the homotrimer, all contribute to a weakening of the mechanical properties of subchondral bone.

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