Phenotypic expression of osteoblast collagen in osteoarthritic bone: production of type I homotrimer

Allen J. Bailey *, Trevor J. Sims, Lynda Knott

Division of Molecular and Cellular Biology, Collagen Research Group, University of Bristol, Churchill Building, Bristol BS40 5DS, UK

Abstract

The metabolism and total amount of the collagen of subchondral bone are increased several fold in osteoathritic femurs compared with controls. We now report for the first time that the quality of the collagen is modified by the formation of type I homotrimer. The homotrimer fibre has been reported to possess a reduced mechanical strength and mineralisation in bone. The presence of the latter therefore accounts for narrower disorganised collagen fibres and decreased mineralisation, and a reduction in mechanical stability of the osteoarthritic femoral head. These changes in the subchondral bone are likely to be of considerable importance in the pathogenesis of osteoarthritis. © 2002 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Bone changes are known to occur in osteoarthritic (OA) joints, with osteophyte formation and subchondral plate sclerosis but have generally received less attention than the articular cartilage. Radin and Rose [1] postulated that the thickening of the subchondral plate would cause stiffening of the bone and as a consequence result in cartilage destruction on loading. An overall increase in cellular activity in OA joints has been detected by technetium scintigraphy, which has also been found to correlate with disease progression as defined by joint space narrowing [2]. The occurrence of these hypertrophic bone changes are not disputed, however, the importance of such changes in the pathogenesis of OA remains contentious and such questions as to why these bone changes occur, and the extent and nature of the mechanisms involved, have not been elucidated.

We have recently shown that the metabolism of bone collagen is much higher in osteoarthritic femoral heads than in age-matched controls in terms of collagen synthesis determined by procollagen peptide assay, increased immature crosslinking, and collagen degradation by increased matrix metalloproteinase and catheptic activity [3,4]. These changes are consistent with increased

* Corresponding author. Tel.: + 44-117-928-9248; fax: + 44-117-928-9505.

E-mail address: a.j.bailey@bristol.ac.uk (A.J. Bailey).

fragility of the subchondral bone collagen [5], which is further compounded by the observed reduction in mineralisation [4].

Further, osteoblasts from OA bone in culture have demonstrated functional and phenotypic differences in comparison with the osteoblasts from normal bone. Westacott et al. [6] reported that the osteoblasts isolated from the subchondral bone of OA subjects are, unusually, capable of degrading the cartilage proteoglycans, whilst Hilal et al. [7] described a markedly altered OA osteoblast expression in vitro in response to increased growth factors. The collagen phenotype expressed by these modified osteoblasts has not been investigated although a change could have a deleterious effect on the structural properties of the bone.

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 molecules in the fibre, hence changes in the molecule and their alignment in the matrix of OA bone have the potential to be detrimental to the mechanical functioning of the tissue. We therefore investigated the phenotypic expression of osteoblasts in OA subchondral bone and demonstrated the presence of a new bone product, type I collagen homotrimer, which compromises the quality of the bone.

2. Materials and methods

2.1. Materials

OA femoral heads, $n = 26$, provided by the Orthopaedic and Rheumatology Departments (University of Bristol), were obtained from elderly (65–90 years) females following hip replacement. Normal femoral heads, $n = 8$, were obtained at post-mortem within 48 h of death. The femoral heads were obtained from the same age range of subjects with no evidence of OA in terms of destruction of cartilage. The individuals had died in hospital or as a result of an accident. None of the individuals had a history of cancer, severe renal or hepatic diseases, nor were they known drug abusers. All specimens were stored at −20 °C. The femoral heads were sliced through the centre to give 2–3 mm sections and punch samples taken from the subchondral bone as described previously [4].

All chemicals, unless otherwise stated, were obtained from Sigma (Dorset, UK).

2.2. Methods

2.2.1. Decalcification of the bone

The bone samples were decalcified prior to analysis for differences in the molecular composition. Bone specimens were snap frozen in liquid nitrogen and pulverised to a fine powder using a stainless steel mill (University of Bristol) which had been precooled in liquid nitrogen. The bone powder was washed with phosphate buffered saline and centrifuged for 5 min at 3000 rpm. The powder was then treated three times with acetone to defat the samples. After air drying the samples were resuspended in tetrasodium EDTA (10% (w/v), 30 mM TRIZMA base and 4 M guanidine hydrochloride at pH 7.5) and extracted for 2 weeks at 4 °C to remove the mineral and non-collagenous proteins. The insoluble collagenous residue was centrifuged, washed free of the EDTA with distilled water and then freeze-dried.

2.3. Molecular composition of bone collagens

2.3.1. (i) Pepsin solubilisation of decalcified bone matrix

The decalcified bone collagen was resuspended in 5 ml of 0.5 M acetic acid and pepsin added to each sample from an enzyme stock in acetic acid to a final concentration of 0.5% w/w of the original dried tissue. Samples were left to stir for 24 h at 4 °C and then centrifuged. The remaining pellet was combined to fresh pepsin and acetic acid (10 ml, 0.5 M) and left to stir for a further 24 h, centrifuged and the supernatant again harvested. Pooled supernatants were freeze-dried and analysed by gel electrophoresis.

2.3.2. (ii) SDS Polyacrylamide gel electrophoresis

The freeze-dried pepsin digested samples were reconstituted at 2.5 mg/ml in buffer (8 M urea, 2 M thiourea, 5% w/v SDS and 25 mM TRIZMA) at pH 6.8. Samples were electrophoresed using a

7.5% polyacrylamide resolving gel matrix employing mini Protean II (Bio-Rad, Hemel Hempstead, UK) apparatus. After staining of the gels with Coomassie Blue and destaining with methanol/acetic acid (10%), band intensities corresponding to the α1 and α2 chains of type I collagen were quantified for ratio analysis of α1:α2. Gels were scanned using an Agfa Studioscan II in transmittance mode using Fotolook SA 2.05 and operated from a Power Macintosh 7100. The images generated were analysed in NIH Image 1.55 allowing quantification of the bands corresponding to α1 and α2 [8].

2.3.3. (iii) Type III collagen

Type III collagen chains were detected on SDS acrylamide gels as a high molecular component (300 kDa), and following delayed reduction of the sample with DTT during electrophoresis the disulphide bonded α1 (III) chains are cleaved and visualised as single type III chains (100 kDa) [9].

2.4. Cyanogen bromide peptide profile

Quantification of the type III content was determined by CNBr cleavage. Decalcified bone collagen (2 mg) was added to CNBr (1 g/ml) in 70% formic acid and incubated at 30 °C for 4 h. The proportion of type I–III was determined from the ratio of α(I)CB8 and α(III)CB8 [10] by determining the band intensities as described above.

2.5. Collagen content

The total collagen content was calculated from the hydroxyproline concentration of the 6M HCl hydrolysate (16 h at 110 °C) determined using the standard colorimetric method employing a continuous autoanalyser (ChemLab, Cambridge, UK) based on the method described by Grant [11]. The colorimetric method was calibrated against the hydroxyproline value determined on the amino acid analyser (Pharmacia).

2.6. Lysine hydroxylation

Samples of the OA and non-OA subchondral bone collagens were acid hydrolysed (6M HCl at 105 °C for 16 h) and analysed for hydroxylysine by standard amino acid composition on an Alpha Plus Autoanalyser (Pharmacia).

2.7. Intermolecular cross-links

The immature (reducible) and the mature (stable) cross-links were determined after borohydride reduction and acid hydrolysis (6M HCl) employing a modified amino acid Alpha Plus autoanalyser (Pharmacia) as previously described in detail [12].

2.8. Differential scanning calorimetry

The thermal denaturation temperatures of the subchondral bone collagen from OA and non-OA subjects were determined by analysis in a Perkin–Elmer DSC-2C fitted with intracoolers and computer controlled at a heating rate of 10 °C/min from 5 to 100 °C using an empty pan as reference. Temperature and energy scales were calibrated with water and indium as standards. The contents of the pans were hydrolysed after denaturation and assayed for collagen by hydroxyproline analysis as described above.

2.9. Electron microscopy

The ultrastructure of the collagen fibres was examined by transmission electron microscopy of the bone compared to age-matched controls. The decalcified bone collagen samples were fixed in 2.5% glutaraldehyde in 0.1 sodium cacodylate pH 7.2 for 24 h, and post-fixed in 1% osmium tetroxide for 1 h. They were then block stained in 1% uranyl acetate for 30 min, dehydrated in alcohol and finally embedded in araldite CY212. After 24 h the blocks were sectioned on a Reichert ultramicrotome and thin sections stained with Reynolds lead citrate and 1% aqueous uranyl acetate before examination in a Philips EM 400.

2.10. Statistical analyses

Data was tested for homogeneity of variances using a two-sample F-test for variances. The sample sizes were not sufficient to test for normality.

For comparisons with equal variances a *t*-test was performed, and for comparisons with unequal variances a non-parametric Mann–Whitney *U*-test was used. Data was analysed using Microsoft Excel and Graphpad Prism.

3. Results

Collagen content. The collagen content of the subchondral bone in the non-OA samples increased from about 80 to 140 µg/g tissue in the OA samples (Table 1).

Molecular composition. Analysis of the molecular composition of the subchondral bone revealed barely detectable levels (less than 5%) of type III collagen in both OA and non-OA subchondral bone.

Table 1
Properties of purified subchondral bone collagen from the femoral head of osteoarthritic and non-osteoarthritic subjects

	OA	Non-OA
Collagen, µg/mg	140 ± 7	79 ± 3*
Hydroxylysine, moles/mole	17.3 (5.7)	10.4 (0.84)**
DHLNL:HLNL ratio	3.0 (0.91)	1.69 (0.59)*
Total immature cross-links, moles/mole	0.50 (0.19)	0.37 (0.17)*
Hyl-Pyr: L-Pyr ratio	3.58 (1.02)	2.96 (0.41)*
Total mature cross-links, moles/mole	0.29 (0.12)	0.35 (0.10)
Denaturation temperature °C	65.7	64.9
Enthalpy J/g	37.3	59.7
$\alpha 1/\alpha 2$ ratio	4:1–17:1	2.4:1

The increase in hydroxylysine content results in changes in the immature and mature cross-link ratios. The samples with the highest $\alpha 1{:}\alpha 2$ ratios also had an increased denaturation temperature and a dramatic decrease in enthalpy. For the biochemical analyses data is presented as means (sd), $n = 12$ for the OA samples, $n = 6$ for the non-OA, **P, 0.01, *P, 0.05. Typical values are presented for the physical data. HLNL, reduced immature cross-link, hydroxylysinonorleucine; DHLNL, reduced immature cross-link dihydroxylysinonorleucine; Hyl-pyr, stable mature cross-link hydroxylysyl pyridinoline; L-pyr, stable mature cross-link Lysyl-pyridinoline.

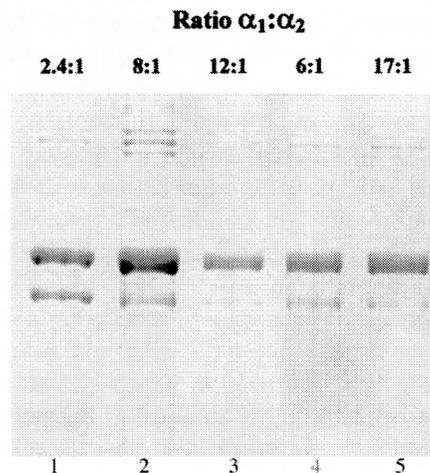**Ratio $\alpha_1{:}\alpha_2$**

Fig. 1. Typical SDS-polyacrylamide gel of subchondral bone collagen from a selection of osteoarthritic human femoral heads examined ($n = 26$) compared to controls. The gels demonstrate the increased ratio of $\alpha 1/\alpha 2$ chains representing an increase in the presence of type I homotrimer replacing the usual type I heterotrimer, compared to non-OA bone collagen. The ratio of $\alpha 1$ to $\alpha 2$ chains was determined by scanning the gels with an Agfa Studioscan II. Control collagen (Track 1) is shown with a ratio $\alpha 1/\alpha 2$ of 2.4:1 employing this technique.

However, there was a general increase in the $\alpha 1/\alpha 2$ ratio in the OA subchondral bone compared with the non-OA samples. The $\alpha 1/\alpha 2$ ratio for the OA subchondral bone varied from 4:1 to 17:1 (Fig. 1) compared to the controls which averaged 2.4:1.

3.1. Thermal stability

Comparison of the thermal stability of the subchondral bone collagen fibres from OA and non-OA samples revealed a slightly higher denaturation temperature (65.7 °C) compared to non-OA bone collagen (64.9 °C) but a decreased enthalpy (that is, the energy required to denature the triple helix) of 37.3 J/g compared to 59.7 J/g for the non-OA bone (Table 1).

3.2. Lysine hydroxylation and cross-linking

Lysine hydroxylation of the OA subchondral bone from the femoral head increased by over 50% compared with non-OA samples (Table 1).

This increase in the level of lysine hydroxylation accounts for the increase in ratios of the immature (DHLNL:HLNL) and the mature (HL-pyr:L-pyr) cross-links. The total immature cross-links were also significantly increased, whilst the total mature cross-links were decreased due to the increase in newly synthesised collagen.

Ultrastructure. The ultrastructure of the collagen fibres in the subchondral bone from the OA subjects were seen to be poorly aligned in the electron microscope compared to the parallel alignment of fibres seen in the control specimens (Fig. 2).

4. Discussion

Our studies on the subchondral bone of OA subjects have revealed, in addition to the increased metabolism and post-translational modifications of the collagen previously reported [4], a change in the phenotypic expression of the os-

teoblasts by the production of a genetically distinct collagen, type I homotrimer. The proportion of the homotrimer, based on the ratio of $\alpha1/\alpha2$, varied considerably, from 4:1 to 17:1, representing a high proportion of the homotrimer in the osteoarthritic bone, compared to 2:1 for the non-OA subjects. The amount of the homotrimer presumably depends on the severity of the OA but further studies on a large number of clinically classified samples will be necessary to confirm this suggestion.

A type I collagen homotrimer has previously been reported in tumours [13] and embryonic [14] derived collagens, but these are thought to be genetically distinct α-chains. The cyanogen bromide cleaved peptide profile of the OA derived collagen showed the same pattern as the heterotrimer collagen from non-OA bone, indicating that the presence of the homotrimer is due to excess 'normal' $\alpha1$-chains. The lysine residues of the $\alpha1$ chain of type I collagen are more hydroxylated than the $\alpha2$ chains. In this study we have
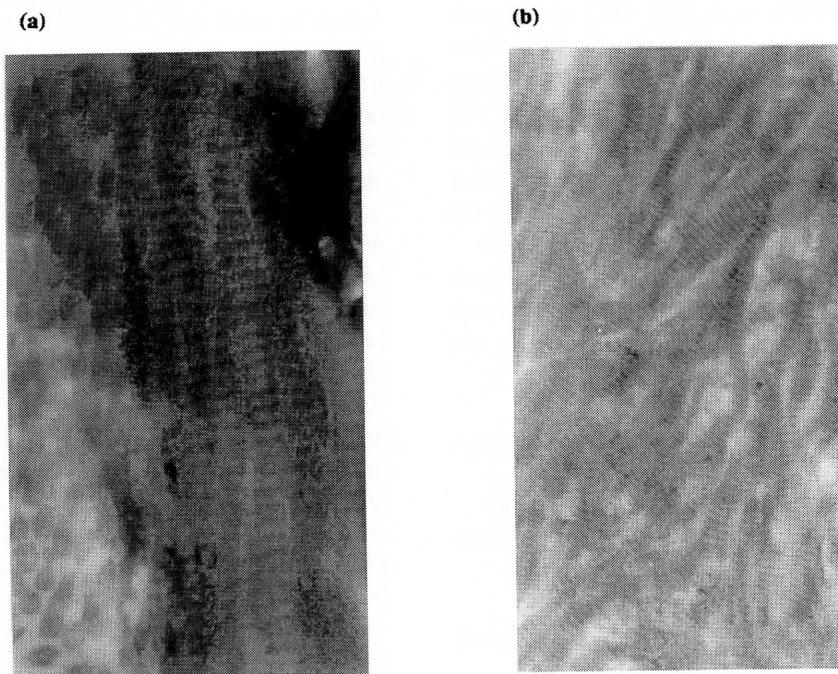
(a)   **(b)**

Fig. 2. Transmission electron micrographs of: (a) normal; and (b) osteoarthritic, bone collagen fibres showing the parallel alignment of the normal bone fibres to the poorly aligned and narrower OA bone fibres.

demonstrated a significant increase in lysine hydroxylation and a concomitant change in the cross-link profile in OA bone compared to non-OA samples (Table 1), which together have the potential to have a significant effect on the physical and biomechanical properties of the bone matrix [15,16].

The stability of the triple helix of the two different collagens was investigated by differential scanning calorimetry [17]. The DSC thermograms demonstrated that the denaturation temperature was slightly higher in the OA samples containing the highest levels of the trimer, but the enthalpy was significantly reduced. The higher denaturation temperature may be due to the small increase in hydroxyproline content of the molecule in the absence of the $\alpha 2$ chain, which contains about 16 residues of hydroxyproline less than the $\alpha 1$ chain. The lower enthalpy of the fibres indicates that the homotrimer molecules are less well packed in the fibre.

We have recently shown a similar effect in a mouse model of osteogenesis imperfecta possessing a deleted $\alpha 2$ chain (unpublished observations). Based on the bone fragility of this form of osteogenesis imperfecta with a deleted $\alpha 2$ chain in humans [18], and the mouse model of OI with a Col $IA2$ gene mutation (oim) [19] one might expect the homotrimer to similarly lead to a decrease in mechanical strength of the OA bone. Indeed Li and Aspden [5] recently reported that the cancellous bone of the human femoral head in OA subjects is mechanically weaker compared with age-matched controls. In the absence of the $\alpha 2$ chain in the oim collagen there is a reduced efficiency for self-assembly resulting in loosely packed collagen fibres [20,21], which in turn leads to decreased strength and abnormal mineralisation [22]. In the present study we have demonstrated that the collagen of OA bone is poorly organised (Fig. 2) and we have previously shown that OA bone is also poorly mineralised [4]. The changes we have described in the subchondral bone of OA subjects; increased lysine hydroxylation, a change in the nature of the intermolecular cross-links, reduced mineralisation, a disorganised collagen matrix, and a change in the thermal properties of the collagen can all be attributed to the presence of the homotrimer. Further more, all these changes are consistent with an increase in the fragility of the subchondral bone of the femoral head.

In view of our identification of the type I homotrimer and its importance in the properties of OA bone we collaborated with Loughlin et al. [23] in a search for a possible association of COL1A1 SP1 polymorphism in OA. Employing a modification of the technique reported by Grant et al. [24] for a COL1A1 association in osteoporosis moderate evidence was obtained for females. Although not statistically significant the result is clearly consistent with increased synthesis of $\alpha 1$ type I collagen and hence the presence of the homotrimer.

The thickened subchondral bone observed in humans is of necessity seen at a late stage of OA and it is not possible to determine whether the bone changes preceded the cartilage degradation or vice versa. However, considerable evidence is accumulating from animal models that subchondral bone thickening clearly precedes cartilage destruction [25]. The most convincing being the studies of Carlson et al. [26] on cynomolgus macaques. Our own studies on the spontaneous OA in the STR/ort mouse [27] and guinea pig [28] similarly revealed thickening of the subchondral bone and an increase in collagen metabolism of the bone.

These changes in the collagenous framework of the human femoral head are clearly worthy of further investigation into their potential role in the progression of OA.

Acknowledgements

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