Collagen cross-linking in human bone and articular cartilage

Age-related changes in the content of mature hydroxypyridinium residues

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The concentration in collagen of hydroxypyridinium cross-linking amino acids was measured in samples of bone and cartilage from human subjects aged from 1 month to 80 years. Cortical and cancellous bone samples were dissected and analysed separately. In both bone and cartilage, the content of this mature form of cross-link reached a maximum by 10–15 years of age (the amount in cartilage being 5–10 times that in bone), then stayed essentially in the same range throughout adult life. In bone the ratio of the two chemical variants of the mature cross-link, hydroxylsylpyridinoline to lysylpyridinoline, was constant throughout adult life at 3.5:1, whereas in cartilage it was always greater than 10:1. The ratio of hydroxypyridinium cross-links to borohydride-reducible keto-amine cross-links also changed with age. The reducible cross-links in bone collagen decreased steeply in content between birth and 25 years, but persisted in significant amounts throughout adult life. Reducible cross-links had virtually disappeared from cartilage by 10–15 years of age, being replaced by hydroxypyridinium residues, their maturation products. Cancellous and cortical bone collagens showed similar trends with age in their content of mature cross-links, though for each subject the concentration in cancellous bone was always lower than in cortical bone, presumably reflecting the higher turnover rate and hence the more immature state of cancellous bone.

INTRODUCTION

Most genetic types of collagen function as cross-linked polymers in the extracellular matrix. The covalent cross-links are formed through the intermolecular reactions of aldehyde residues made on the protein monomers by lysyl oxidase (Piez, 1968; Tanzer, 1976; Light & Bailey, 1980; Robins, 1983). For the familiar fibril-forming collagens two cross-linking pathways can be defined, one based on precursor lysine aldehydes, the other on precursor hydroxysine aldehydes (Eyre et al., 1984a). The lysine aldehyde pathway dominates in mature skin, and the hydroxysine aldehyde pathway in bone, cartilage and most other major connective tissues of the body. The initial cross-links on both pathways are borohydride-reducible aldimines (or their keto-amine re-arranged products) whose structures and tissue distribution are well documented. These reducible cross-links diminish greatly in concentration as connective tissues mature, it is thought by further spontaneous reactions within the collagen polymer to form mature non-reducible compounds (Bailey & Shimokomaki, 1971; Robins et al., 1973). The structures of the mature contents on the lysine aldehyde pathway are still uncertain (Barnard et al., 1987; Mechanic et al., 1987), but on the hydroxysine aldehyde pathway hydroxypyridinium residues have been identified as mature products that form from the intermediate keto-amines in most tissues (Eyre et al., 1984a).

One report on human cartilage and tendon showed that the hydroxypyridinium cross-links increase in concentration as the tissues grow and mature, but the limited measurements suggested that they may fall again with increasing adult age (Moriguchi & Fujimoto, 1978). This implied that these 'mature' cross-links may actually be intermediate on a more complex pathway. Other findings on bovine bone, cartilage and human dentine, however, showed that hydroxypyridinium cross-links remain prominent after maturity, and so could be considered to be mature cross-linking structures in collagen fibrils (Eyre et al., 1981; Walters & Eyre, 1983; Eyre et al., 1984a). Nevertheless, in bone and dentine, unlike cartilage, the reducible residues also persisted as prominent cross-links even in the adult tissues. A delayed maturation of reducible cross-links in the calcified collagens of bone and dentine compared with uncalcified collagen was indicated from incubation experiments in vitro (Eyre, 1981).

In the present study, we measured the contents of both the borohydride-reducible and the mature hydroxypyridinium cross-linking amino acids in samples of human bone and cartilage from subjects aged 0–80 years. The aims were to confirm the persistence of hydroxypyridinium residues as mature cross-links throughout life and to seek any age-related variations in their concentration.

MATERIALS AND METHODS

Tissue source and preparation

Bone and articular cartilage were dissected from femurs obtained during post-mortem examination of 27 subjects who had died from accidental causes or from
medical causes other than renal disease or cancer. Full-thickness articulating cartilage was sliced with a scalpel from the epiphyses, avoiding the joint margins, and freeze-dried. The femoral diaphysis was cleaned of adhering soft tissues, split longitudinally, and marrow and trabecular bone were removed from the endosteal surface. The bone was washed briefly with methanol, chloroform/methanol (2:1, v/v) and finally methanol before being air-dried, broken into small pieces and reduced to a fine powder in a freezer mill (Spex Industries) cooled with liquid N₂. Pieces of cancellous bone were obtained from the femoral epiphyses by using a handsaw and an osteotome and pulverized in the freezer mill. The powdered tissue was washed by suspension in 0.15 M-NaCl (6 ml/g of frozen powder), followed by low-speed centrifugation for 5 min. The supernatant solution was discarded and the procedure repeated on the residue. Bone powder was demineralized at 4°C by extraction for 4 × 3 days with 0.5 M-EDTA (pH 7.6, containing 1 M-phenylmethylasulphonyl fluoride and 1 M-iodoacetic acid as proteinase inhibitors; 15 ml/g wet wt. of tissue). The collagenous residue was finally washed for 2 × 30 min with water before freeze-drying. Cartilage and bone samples were stored at −20°C before analysis.

Reagents

All reagents and solvents were of the best analytical or h.p.l.c. grades available.

Assay of borohydride-reducible and mature cross-linking amino acids

Portions (200 mg) of decalcified cortical bone powder were stirred in 0.1 M-sodium phosphate, pH 7.5, at room temperature and 10 mg of NaBH₄ was added. After 30 min, a further 10 mg of NaBH₄ was added. Excess NaBH₄ was destroyed after 60 min by lowering the pH below 4 with acetic acid. The bone matrix particles were recovered by low-speed centrifugation, washed with water and freeze-dried. This material was hydrolysed in 6 M-HCl in sealed glass tubes at 108°C for 24 h. The dried hydrolysate (Buchler Evapomix; Haake Buchler, Saddle Brook, NJ, U.S.A.) was eluted from a calibrated column (90 cm × 2.5 cm) of Bio-Gel P2 (200–400 mesh; Bio-Rad Laboratories, Richmond, CA, U.S.A.) in 10% (v/v) acetic acid at room temperature, to separate the pool of collagen cross-linking amino acids from the bulk amino acids (Eyre, 1981). The elution positions of the hydroxyproline and amino acids were established by their fluorescence, and those of the borohydride-reduced cross-links by using ³H-labelled standards (Eyre, 1987). Fractions were pooled and freeze-dried to recover quantitatively all the cross-linking amino acids, from which a sample was analysed to measure the four individual cross-linking residues, HP, LP, DHLN and HLN, by ion-exchange chromatography and ninhydrin detection on a Glenco modular amino acid analyser (Eyre, 1987).

Assay of hydroxyproline cross-links by reverse-phase h.p.l.c.

A direct h.p.l.c./fluorescence assay (Eyre et al., 1984b) was used to measure the two forms of hydroxyproline cross-link (HP and LP) in all samples of bone and cartilage. This method could be applied directly to hydrolysates of crude tissue, including mineralized bone, without an intermediate clean-up step by molecular sieve chromatography.

Hydroxylysine and hydroxyproline analyses

The hydrolysates of mineralized cortical bone were assayed for hydroxylysine and hydroxyproline by using a Beckman 121 MB automated amino acid analyser equipped with post-column ninhydrin detection. Specific hydroxyproline analyses were also made on portions of all tissue hydrolysates by using the colorimetric assay of Stegemann (1958) adapted to the Technicon Auto-analyzer.

RESULTS

Bone collagen

Fig. 1 shows an ion-exchange elution profile for the four cross-linking amino acids recovered from a sample of borohydride-treated human bone collagen. The age-related changes plotted in Fig. 2 were derived from such analyses on the series of cortical bone specimens from individuals aged 5 months to 80 years. A steady decrease is evident in the content of the total reducible cross-links from birth until about 25 years of age. In contrast, the content of the mature cross-links, HP plus LP, shows an increasing trend from birth to 25 years (linear-regression analysis gives a slope from 0.2 to 0.4 mol of HP plus LP/mol of collagen, with a correlation coefficient of 0.92). Thereafter, the contents of both types of cross-link level off, with a downward trend still evident for the reducible compounds and perhaps also a slight fall with age emerging in the content of the mature residues, although the latter could not be proved with statistical significance. The ratio of reducible cross-links, DHLN/HLN, and the ratio of mature cross-links, HP/LP, also changed with age, as shown in Fig. 3. Again the most significant trend is a fall in both ratios between birth and skeletal maturity.

Additional portions of the cortical bone powder
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Fig. 2. Age-related changes in the content of reducible and mature cross-linking residues in human bone collagen

Samples of cortical bone were analysed as described in Fig. 1. Collagen in mol was calculated from a hydroxyproline analysis on the initial hydrolysate.

Fig. 3. Age-related changes in the ratios of the two forms of reducible cross-link and the two forms of mature cross-link in human bone

Samples of cortical bone were analysed as described in Fig. 1.

were hydrolysed directly in 6 M-HCl, without prior demineralization for analysis of cross-linking residues directly by the h.p.l.c./fluorescence method. Samples of cancellous metaphyseal bone taken from the same femur were analysed similarly. A typical chromatographic elution profile is shown in Fig. 4(a). The results of these analyses are plotted in Fig. 5. The trends with age shown for HP and LP by the two-stage method of cross-link analysis on cortical bone appeared to be even more pronounced for cancellous bone. Thus the increase over the first decade of life and the downward trend after about 20 years of age appeared steeper than for cortical bone. There were too few data points to apply meaningful statistics. However, for every individual (except one; Fig. 5) the concentration of hydroxyprolineum cross-links in collagen was higher for cortical bone than for cancellous bone (at a mean ratio for cortical/cancellous of 1.6 ± 0.5; mean ± S.D., n = 23). No significant difference emerged from these data between males and females.

Fig. 6 plots for cortical bone the change with age in the summed concentration of all the cross-links. Each measurement was expressed as lysine equivalents of ninhydrin colour from the amino acid analyser, as derived from the raw data used to plot Fig. 2. This index of overall cross-linking shows a marked fall between birth and > 50 years, reaching a value about one-third that seen in the newborn. Over the same period, the hydroxlysine content of the bone collagen also shows a gradual decrease from five to four residues per 100 residues of hydroxyproline that statistically is highly significant (Fig. 7).

Cartilage collagen

Only mature hydroxyprolyridinium cross-links were measured in the complete series of specimens of articular cartilage (Fig. 8). A typical chromatographic analysis of the HP residues in hydrolysed cartilage is shown in Fig. 4(a). By running samples at even higher loads, the molar ratio of HP to LP was found to be about 50:1. Previous analyses of mature articular cartilage from various species had shown very low amounts of reducible cross-links relative to the mature HP cross-links. Two samples of human articular cartilage that were put through the full analysis procedure described above gave the following results: 16-year-old female, 2.3 mol of HP to < 0.2 mol of DHLN per mol of collagen; 23-year-old male, 1.9 mol of HP to 0.14 mol of DHLN per mol of collagen, each cross-linking residue being expressed as lysine molar equivalents by ninhydrin colour on the amino acid analyser.
Measurements were made as described in Fig. 4 on pairs of samples from 23 individuals. Each data point is plotted as the sum of HP and LP residues per collagen molecule. Symbols: ●, ◆, male; ○, △, female; ●, ○, cortical bone; ◆, △, cancellous bone.

The concentrations of the four cross-linking amino acids measured as described in Fig. 1 were each expressed as lysine molar equivalents of ninhydrin colour and summed for each bone sample. The ordinate units are therefore molar equivalents per mol of collagen. Symbols: ◆, male; △, female. The smooth curve drawn through the points is the best fit generated by the least-squares method and a second-order polynomial equation.

The results plotted in Fig. 8 show that the content of mature HP residues increases in the cartilage collagen during skeletal growth, reaching a maximum in the 15–25-year age group. (By linear-regression analysis the slope of the increase from birth to 24 years went from 1 to 2 mol of HP/mol of collagen, had a correlation coefficient of 0.94 and was highly significant, P < 0.01.) Thereafter it stays at a similar value throughout life, in the range 1–2 mol of HP/mol of collagen. A slight downward trend is evident between skeletal maturity and old age (down by 12% between 24 and 80 years by linear-regression analysis, with a correlation coefficient of 0.44). The results also show that the pyridine cross-links are 5–10 times more concentrated in mature cartilage collagen than in mature bone collagen.

DISCUSSION

The findings establish that 3-hydroxypyridinium cross-linking amino acids persist in human bone and cartilage collagens throughout adult life. A previous report on a limited number of samples of human costal cartilage (Moriguchi & Fujimoto, 1978) had suggested that these residues rose and fell in concentration quite...
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Collagen rapidly during growth and maturation, implying that they were intermediate, not final, cross-links. In cartilage, the rapid decrease in concentration of reducible cross-links and the increase in 3-hydroxypyridinium cross-links pointed to a direct precursor–product relationship (Eyre et al., 1981), and indicated a stoichiometry whereby two residues of the difunctional reducible keto-aminе gave rise to one difunctional 3-hydroxypyridinium residue (Eyre & Oguchi, 1980). The apparent half-life of reducible cross-links in the reaction to form mature cross-links was about 2 weeks, based on [H]lysine labelling of rabbit cartilage in vivo (Eyre et al., 1981). In bone, the present findings and previous findings with bovine bone (Eyre, 1981) suggest that a different maturation pathway may also operate.

Interpreting the results of the cross-linking analyses on bone specimens is complicated by the continual remodelling (resorption and new deposition) that occurs in bone throughout life. The differences in cross-linking contents between cancellous bone from the metaphysis and cortical bone from the diaphysis may reflect this process (Fig. 5), since cancellous bone is believed to remodel more rapidly than cortical bone (Marshall et al., 1973). Hence recently made bone collagen, rich in immature cross-links, will be present to some degree throughout life. Nevertheless, this cannot be the sole explanation for the persisting reducible cross-links. The chemical pathway of cross-linking in bone collagen also appears to be arrested or inhibited at the stage of the reducible bonds by the mineralization process. Thus the rate of maturation in vivo of reducible cross-links to mature cross-links was much slower in mineralized than in demineralized samples of young bovine bone (Eyre, 1981). This effect of mineralization on the collagen fibrils may be one reason for the relatively low natural content of hydroxypyridinium cross-links in bone collagen.

In addition, however, the age-dependent data reported here (Figs. 2 and 6) clearly show that a significant fraction of the reducible cross-links must also disappear from bone collagen with time through another mechanism, i.e. not to hydroxypyridinium residues. Apparently some other product is forming that has not yet been identified. The rate of this latter unidentified reaction must be very slow, however, apparently taking years (Fig. 2) rather than the short time scale of days to a few weeks needed for the reducible residues to be converted into the hydroxypyridinium residues in cartilage and other non-mineralized connective tissues that primarily use the hydroxysine aldehyde pathway (Eyre et al., 1984a).

One speculative possibility, for which indirect data have been provided from other tissues, is a slow oxidation of the reducible aldime cross-links by molecular oxygen to form amides (Bailey et al., 1977). Assuming that the bone collagen fibrils become dehydrated by the mineralization process, as neutron-diffraction data have suggested (Bonar et al., 1985), this may restrict molecular fluctuations and side-chain movements that presumably are needed to permit the cross-link maturation reaction that forms HP and LP residues, in which case the ‘fossilized’ reducible cross-links may then with time be oxidized and so become undetectable by the present methods. Alternatively, other mature (non-reducible) cross-links may form from the aldime/keto-amine cross-links. Barnard et al. (1987) have described a suspected cross-link (compound M) derived from lysine in skin, which they report is present in bone, but no concentrations are given. Mechanic et al. (1987) describe what may be the same compound in skin and identify it as histidinohydroxysinonorleucine instead of the hydroxylaladhistidine structure previously suggested (Housley et al., 1975). They note, however, that this compound is not present in skeletal tissues.

The reason for the slight decrease in concentration of HP and LP residues during adult life is unknown. Loss owing to unknown chemical reactions is one possibility; another is that collagen made in an older individual may be less cross-linked (for instance, if cell-regulated activity of lysyl oxidase decreased with age), and so through natural turnover of bone the mean number of cross-links would fall. This latter concept is supported by the steeper fall with age in the mature cross-links of cancellous bone, with its higher turnover rate, compared with cortical bone (Fig. 5).

The LP form of mature cross-link is formed in significant amounts only in the mineralized collagens of bone and dentine (Eyre et al., 1984a,b), whereas HP is widely distributed among tissue types. The HP/LP ratio in bone varies considerably among species (from 10:1 to less than 2:1). The present results establish that the ratio is fairly constant in human bone at 3–4:1. This ratio could therefore be a characteristic diagnostic marker for degradation products of bone collagen in body fluids.

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