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ORIGINAL ARTICLE

Reductions in degree of mineralization and enzymatic collagen cross-links and increases in glycation-induced pentosidine in the femoral neck cortex in cases of femoral neck fracture

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Abstract *Introduction:* Enzymatic and glycation-induced nonenzymatic cross-links play important roles in the expression of bone strength. The cross-link pattern is affected by tissue maturation and senescence. The aim of our study was to understand the distinctive posttranslational modifications of collagen in areas with different degrees of mineralization with and without hip fracture. *Methods:* Sixteen female cases of intracapsular hip fracture (78±6 years) and 16 age- and gender-matched postmortem controls (76±6 years) were included in this study. A sample of each femoral neck cortex was fractionated into low (1.7 to 2.0 g/ml) and high (>2.0 g/ml) density portions. The contents of enzymatic cross-links (dihydroxylysinonorleucine, hydroxylysinonorleucine, lysinonorleucine, pyridinoline, and deoxypyridinoline) and nonenzymatic cross-links (pentosidine) and the extent of lysine (Lys) hydroxylation were determined in each fraction. *Results:* In the controls, there was no significant difference in the contents of enzymatic cross-links between low- and high-mineralized bone fractions whereas pentosidine content was significantly higher in high-mineralized bone compared with low-mineralized bone (p=0.0014). When comparing enzymatic cross-link contents between controls and fracture cases, a trend toward lower (p=0.0961) cross-link content in low-mineralized bone and a significant reduction (p<0.0001) in high-mineralized bone were observed. Pentosidine content of low-mineralized bone was significantly higher in fracture cases than in controls (p<0.0001). The extent of Lys hydroxylation was significantly higher in fracture cases than in controls (p<0.001). The higher hydroxylation of Lys in collagen from fracture cases relative to controls was associated with significantly higher values of hydroxylysine-derived cross-link such that the enzymatic cross-link patterns correlated with the extent of Lys hydroxylation in the collagen molecules. *Conclusions:* These results suggest that reductions in the degree of mineralization and enzymatic cross-links and excessive formation of pentosidine may play an important role in explaining poor bone quality in osteoporosis.

Keywords Bone quality · Cortical bone · Cross-links · Degree of mineralization · Osteoporosis · Pentosidine

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Introduction

Bone loss and impaired bone quality, which encompass the structural and material properties of bone, have been proposed as major causes of increased bone fragility in osteoporosis [1–3]. The material properties of bone are thought to be influenced by the degree of mineralization of basic structure units (BSUs), microdamage accumulation, and collagen cross-link formation [4–7]. Thus, the degree of mineralization of each BSU depends on BSU lifespan as regulated by the turnover rate [8, 9]. Depending on turnover rate, bone tissue exhibits heterogeneously varying degrees of mineralization in different areas [8, 10, 11]. Hence, the material properties of newly synthesized matrix may differ in tissue maturity and senescence from older matrix. Additionally, newly synthesized matrix in osteoporotic patients may not necessarily be as well-made as in age-matched nonosteoporotic subjects, and the osteoblastic and osteocytic functions and pericellular environment in osteoporotic patients differ from those in nonosteoporotic subjects [12, 13].

Data have accumulated that collagen cross-links play important roles in the expression of bone strength [6, 14–17] and the proper biological function of bone [18–23]. The cross-links of collagen can be roughly divided into enzymatic types, such as lysyl-oxidase-mediated cross-links, and nonenzymatic types, such as advanced glycation end products (AGEs) according to the mechanism of formation and functional differences. Enzymatic cross-links are formed initially as divalent immature ketoimines via the

action of the enzymes lysyl oxidase and lysyl hydroxylase that undergo spontaneous reaction to form a trivalent mature pyridinium cross-link [19, 22]. Oxlund et al. demonstrated that impaired enzymatic cross-link formation is coincident with a decrease in bone strength [14, 15]. Thus, the enzymatic cross-links are considered to be a beneficial class because they have a positive effect on the physiological functions of bone. Several investigations concerning enzymatic cross-links have focused only on mature cross-links, including pyridinoline (Pyr) and deoxypyridinoline (Dpyr), without accounting for the presence of immature cross-links consisting of dehydrodihydroxylysinonorleucine (deH-DHLNL), dehydrohydroxylysinonorleucine (deH-HLNL), and dehydrolysinonorleucine (deH-LNL). However, immature as well as mature cross-links may affect the physiological function of bone as well as the other connective tissues [14, 24–26]. These observations have led to the proposal that a simultaneous estimate of both immature and mature cross-links is important for elucidating the actual dynamic state of enzymatic cross-link formation. It is generally thought that not only quantitative changes in enzymatic cross-links but also the degree of hydroxylation of lysine (Lys) involving crucial cross-linking sites, which is regulated by Lys hydroxylases, would be associated with the alteration of collagen and biological and mechanical functions of bone [27–29]. Thus, both cross-link formation and the extent of Lys hydroxylation should be measured simultaneously to determine the quality of collagen in bone.

AGEs, which are formed by a nonenzymatic glycation [17] or oxidation reaction [30], are associated with tissue maturation and subsequent senescence [25, 31]. In contrast to the positive effects bestowed by enzymatic cross-links, nonenzymatic cross-links have detrimental effects on the mechanical [6] and biological [32] functions of bone. Accumulation of nonenzymatic cross-links in bone decreases postyield properties and toughness and makes collagen fibers brittle [6, 17]. Thus, AGEs are considered to be a class of disadvantageous cross-links in bone.

Both enzymatic and nonenzymatic cross-link formation are posttranslational processes that are influenced mainly by tissue maturation time, extracellular environment, and the secondary mineralization process described above. Because the distinctive collagen reactions may be carried to different degrees of mineralization in different bone areas, we should analyze separate fractions from an area, each containing a sample with a different degree of mineralization. However, the classical whole-bone analysis cannot be used to estimate different degrees of mineralization in certain areas. In the present study, the technique of density gradient fractionation developed by Russell et al. [11] and refined by Grynpas et al. [10] was used to obtain the separation of BSUs in different stages of mineralization.

The aim of the present study was to compare the degree of mineralization and collagen posttranslational modifications in femoral neck fracture cases and age- and gender-matched controls.

Materials and methods

Subjects

Cortical bone specimens were obtained from 16 female intracapsular hip fracture cases aged 72–86 (mean 78±6) years. Cortical bone from a complete cross-section from each patient undergoing elective surgery was sampled 5 mm away from the bone surface, which had been sawn at hemiarthroplasty, to ensure that the tissue was not heat damaged. In order to exclude the effect of fracture healing, patients were operated on 2–4 (mean 2.4) days after the fracture. The age- and gender-matched, nonfractured, control samples from 16 female subjects aged 69–89 (mean 76±6) years were obtained postmortem from the equivalent site of the femoral neck within 24 h after an accidental death. The cases with hip fracture and control subjects were excluded who had osteoarthritis or preexisting conditions known to affect bone metabolism, such as diabetes, renal failure, and rheumatoid arthritis. Patients and control subjects were not considered to be emaciated or obese; their body mass indexes (BMIs) were within the range 21–25 kg/m^2.

Informed consent to use this material for research was given at the time of surgery by the patients themselves, according to procedures required by the distinct ethics committee and the hospital trust. Bone specimens of control subjects were taken from samples used in our previous study into age-related changes in biochemical characteristics of collagen from human subjects, and their use was with the consent of relatives and was approved by the distinct ethics committee [33].

Density fractionation

The bone was cleaned of adhering soft tissues and marrow. Bone specimens were pulverized in liquid nitrogen and sieved in a sonic sifter to isolate bone particle sizes below 20 μm. Recovery after grinding and sieving was 95% by weight. The bone powder was defatted with chloroform/methanol (2:1, v/v) for 48 h and then methanol for 12 h before being air dried. The bone powder was fractionated into three fractions corresponding to the osteoid (<1.7 g/ml) [34, 35], low- (1.7–2.0 g/ml), and high- (>2.0 g/ml) density fractions in a bromoform-toluene mixture by the stepwise centrifugation method of Grynpas [36]. The osteoid fraction contained only a trace level of the mineral phase in both normal and fracture samples; therefore, no further analysis for the biochemical nature of collagen was done for this fraction. Biochemical analyses were carried out on the high- and low-density bone fractions. We defined the boundary line between the low- and high-density fractions at 2.0 g/ml based on reports by by Sodek et al. [35]. They showed that the distribution of bone particles after density fractionation showed a peak at a density of 2.0 g/ml. By separating bone at that boundary line of 2.0 g/ml, we obtained sufficient bone from both low- and high-density fractions for triplicate analyses of collagen posttranslational modifications.

Approximately 200 mg of sifted bone powder was added to a polyallomer tube containing 30 ml of 2.0 g/ml-density solution (calibrated with sink float). The bone powder suspension was centrifuged at 10,000 g for 30 min. Density of the supernatant was then adjusted to 1.7 g/ml by the addition of toluene, and the new suspension was recentrifuged. Contents of each fraction were recovered after adding about 10 ml of toluene and subsequent centrifugation for 30 min at 6,000 g. The powders were washed in three changes of toluene and allowed to dry at room temperature. The material in the fraction was weighed, and the weight fraction of the low-density material relative to total dry bone was calculated and expressed as a percentage.

To determine whether toluene and bromoform affected the biochemical nature of bone collagen, two equal amounts of bone powder were compared; one sample was exposed to bromoform-toluene, and the other was not. One hundred milligrams of human bone powder was kept in a bromoform-toluene mixture at 5°C for 6 h then centrifuged after addition of toluene to obtain a clear supernatant. The pellet was washed in toluene and lyophilized. The control sample was treated identically except for the exposure to bromoform-toluene. The amounts of collagen and cross-links were determined as described below. It was found that these quantities were identical in the treated and control samples.

Calcium and phosphorus contents in bone

Calcium and phosphorus concentrations of the low- and high-density fractions were analyzed as follows. Approximately 100 mg of each fractionated bone powder was dried at 105°C for 24 h, weighed, and ashed at 700°C for 5 h. Then, calcium and phosphorus concentrations of the specimens were determined by atomic emission spectroscopy (ICP-AES, Nippon Jarrell-Ash Co., Ltd, Kyoto, Japan) after the organic matrix was removed by ignition and oxidation in a mixture of perchloric and nitric acids [37]. The amounts of calcium and phosphorus are expressed as percent of bone dry weight.

Collagen content in bone

An aliquot of each bone powder was weighed and hydrolyzed in 6 M hydrochloric acid at 110°C for 24 h. The amount of collagen in bone was determined from the hydroxyproline (Hyp) content measured by high-performance liquid chromatography. It was assumed that collagen weighed 7.5 times the measured Hyp weight, with a molecular weight of 300,000 [38]. Total collagen content was expressed as a dry weight percentage relative to the original powdered bone tissue. The resulting data were then used to calculate the cross-link values as molar/molar of collagen [25].

Characterization of enzymatic and nonenzymatic collagen cross-links

The reduction of collagen in bone with sodium borohydride ($NaBH_4$) (Sigma-Aldrich, St Louis, MO, USA) and measurement of cross-links were carried out as previously described [25]. Briefly, each bone powder was demineralized twice with 0.5 M EDTA in 50 mM Tris buffer, pH 7.4, for 96 h at 4°C. Demineralized bone residues were then suspended in potassium phosphate buffer, pH 7.6 (0.15 ionic strength), and reduced at 37°C with $NaBH_4$. The reduced specimens were hydrolyzed in 6 N hydrochloric acid at 110°C for 24 h. Hydrolysates were then analyzed for cross-links on a Shimadzu LC9 high-performance liquid chromatography (HPLC) fitted with a cation exchange column (0.9×10 cm, Aa pack-Na, JASCO, Ltd., Tokyo, Japan) linked to an on-line fluorescence flow monitor (RF10AXL, Shimadzu, Shizuoka, Japan).

We determined the content of enzymatic immature reducible and mature nonreducible cross-links and nonenzymatic cross-links such as the AGE pentosidine (Pen). Lysyl-oxidase-mediated reducible immature cross-links (deH-DHLNL, deH-HLNL, and deH-LNL) were identified and quantified according to their reduced forms (DHLNL, HLNL, and LNL, respectively). Our established HPLC system enables us to determine enzymatic and nonenzymatic cross-link contents within a linear range from 0.2 to 600 pmol in bone specimens. Reducible immature cross-links and common amino acids, such as Hyp, hydroxylysine (Hyl), and Lys, were detected with o-Phthalaldehyde derivatization by the postcolumn method whereas enzymatic nonreducible mature cross-links, such as Pyr, Dpyr, and the nonenzymatic glycation-induced cross-link Pen, were detected by natural fluorescence. Contents of each cross-link were expressed as mole/mole of collagen.

Lysine hydroxylation of collagen

The level of Lys hydroxylation in the α1 and α2 chains of type I collagen in bone, including or excluding telopeptides, was analyzed according to Uzawa et al. [19] with some modifications [21]. To estimate the level of Lys hydroxylation in the collagen molecules, including both telopeptides and helical domains, decalcified samples were extracted in 0.5 N acetic acid for 2 days at 4°C with gentle agitation. Samples were then centrifuged at 20,000 g for 30 min, and the supernatants were lyophilized (acid-soluble fraction). For the analysis of Lys hydroxylation in collagen molecules that excluded telopeptides, pepsin-extractable collagen was isolated by digesting the acid-soluble fraction with pepsin (Worthington, Freehold, NJ, USA) at an enzyme:substrate ratio of 1:10 in 0.5 M acetic acid (substrate content 10 mg/ml) at 5°C for 48 h. After digestion, residues were removed by centrifugation at 20,000 g for 30 min, NaCl was added to the supernatants to a final concentration of 0.7 M, and the mixture was stirred for 24 h. Precipitates were collected by centrifugation at 25,000 g for 30 min, dialyzed against 0.5 N acetic acid, and

then lyophilized. Acid-soluble fractions and pepsin-extractable fractions were subjected to 5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions, transferred onto Trans-Blot Transfer membranes (BioRad Laboratories, Inc., Hercules, CA, USA.), and stained with 0.25% Coomassie Brilliant Blue (Wako, Tokyo, Japan). Visualized bands corresponding to the α1 and α2 chains of type I collagen were excised, hydrolyzed, and subjected to amino acid analysis with established methods [25]. Purified proteins were identified as α1 and α2 type I collagen chains by comparing with respective standards prepared from bovine skin type I collagen with or without pepsin treatment (KOKEN, Tokyo, Japan) and assessing amino acid composition. We confirmed that the purified collagen consisted of Gly in the range of 322 to 333 residues per 1,000 amino acids and greater than 80 residues Hyp per 1,000 amino acids, which is in accord with the known amino acid composition of type I collagen. The extent of Lys hydroxylation was expressed as the ratio of Hyl/(Lys+Hyl) [19, 21–23].

Statistical analyses

All values are listed as means with their standard deviation (mean±SD) in the text and the tables. Box and whisker plots to show the distribution of variables were constructed for the cross-links in controls and fracture cases. Where the data were normally distributed, statistical significance was determined by one-way analysis of variance (ANOVA) with post hoc adjustment for multiple comparisons with the JPM statistical software package (V.3.1.6, SAS Institute, Cary, NC, USA). Where data were not normally distributed, normalization by log transformation preceded the above and was successful in each case. Differences in cross-link parameters between low- and high-density fractions within each case were analyzed with a paired *t* test.

Results

Density fractionation and mineral contents in low- and high-density fractions

The proportion of the low-density fraction relative to total bone (w/w, %) in bone from the fracture cases was significantly higher than that from the controls (fracture cases, 43.6±22.3%; controls, 14.9±10.3%; p=0.00339). The calcium and phosphorus contents and the Ca/P ratio of the low-density fraction were significantly lower than those of the high-density fraction in both the controls (calcium, p=0.0002; phosphorus, p<0.0001; Ca/P, p<0.0001) and the fracture cases (calcium, phosphorus, and Ca/P, p<0.0001) (Table 1). The contents of calcium and phosphorus of the low-density fraction from the fracture cases were significantly lower than those from the controls (p=0.0011 and 0.0005, respectively) while there were no significant differences in calcium and phosphorus contents of the high-density fraction between controls and fracture cases.

Enzymatic and nonenzymatic cross-links in bone

Controls and the fracture cases had similar bone collagen contents as a percent of tissue dry weight (28.9 and 31.8%). There were also no significant differences in the collagen contents between the low- and high-mineralized bone fractions from the two groups (Table 2).

In controls, there was no significant difference in the content of total enzymatic cross-links (the sum of DHLNL, HLNL, LNL, Pyr, and Dpyr) between low- (0.929±0.110 mol/mol of collagen) and high- (0.907±0.092 mol/mol of collagen) mineralized bone fractions (Table 2 and Fig. 1a) whereas the content of Pen nonenzymatic cross-links was significantly higher in high-mineralized bone than in low-mineralized bone (p=0.0014) (Table 2 and Fig. 1b). In fracture cases, a trend (p=0.03) toward lower enzymatic cross-link content of high-mineralized bone fraction (0.670±0.112 mol/mol of collagen) was observed compared with that of low-mineralized bone fraction (0.842±0.195 mol/mol of collagen) (Table 2 and Fig. 1a) while the nonenzymatic cross-link, Pen, content of low-mineralized bone fraction was significantly higher

Table 1 Calcium (Ca) and phosphorus (P) contents and Ca/P ratios in the femoral cortex of fracture cases and controls

	Calcium (mg/g of tissue dry weight)	Phosphorus	Ca/P
Controls			
Low density	227.6 ± 6.2	103.6 ± 2.8	2.198 ± 0.022
High density	245.9 ± 1.7[b]	111.7 ± 0.6[b]	2.204 ± 0.019[b]
Fracture cases			
Low density	206.1 ± 19.7[a]	91.7 ± 9.9[a]	2.250 ± 0.037
High density	241.5 ± 2.2[b]	107.9 ± 0.9[b]	2.238 ± 0.023[b]

Values are expressed as mean±SD
[a]Significant differences between controls and fracture cases within the same density (low or high) at p<0.0001
[b]Significant differences between low- and high-density fractions within the same individuals at p<0.0001

Table 2 Comparison of contents of enzymatic and nonenzymatic cross-links between controls and fracture cases

	Collagen contents (% of tissue dry weight)	Enzymatic cross-links					Nonenzymatic cross-link
		Immature reducible forms (mol/mol of collagen)			Mature nonreducible forms (mol/mol of collagen)		AGEs (mmol/mol of collagen)
		DHLNL	HLNL	LNL	Pyr	Dpyr	Pen
Controls							
Low density	30.6±2.1	0.388±0.089	0.243±0.036	0.071±0.012	0.168±0.017	0.059±0.009	4.147±2.470
High density	31.8±1.4	0.398±0.057	0.213±0.041	0.068±0.008	0.171±0.020	0.057±0.007	7.818±3.452
Fracture cases							
Low density	29.2±2.8	0.343±0.090	0.253±0.083	0.038±0.008[c]	0.175±0.036	0.034±0.009[c]	24.069±16.742[c]
High density	28.9±3.6	0.315±0.062[a]	0.161±0.044[a]	0.042±0.010[c]	0.128±0.048[b]	0.024±0.011[c]	13.297±8.040

Values are expressed as mean±SD
AGE advanced glycation end products, *DHLNL* dihydroxylysinonorleucine, *HLNL* hydroxylysinonorleucine, *LNL* lysinonorleucine, *Pyr* pyridinoline, *Dpyr* deoxypyridinoline, *Pen* pentosidine
[a,b,c]Significant differences between controls and fracture cases within the same density group (low or high) at $p<0.05$, $p<0.01$, and $p<0.0001$, respectively

than that of high-mineralized bone fraction ($p=0.006$) (Table 2 and Fig. 1b).

When comparing the contents of enzymatic cross-links in controls and fracture cases, a trend toward lower ($p=0.0961$) cross-link content in low-mineralized bone and a significant reduction ($p<0.0001$) of cross-link content in high-mineralized bone were observed in fracture cases (Fig. 1a). Pen content of low-mineralized bone fractions was significantly higher in fracture cases than in controls ($p<0.0001$). Although Pen content in high-mineralized bone fractions from fracture cases increased by 80% compared with the same fraction from controls, this difference did not reach statistical significance (controls, 7.818±3.452 mmol/mol of collagen; fracture cases, 13.297±8.040; $p=0.1226$) (Fig. 1b).

Estimates of collagen maturity and senescence

Ratio of the content of total nonreducible mature pyridinium cross-links (Pyr+Dpyr) to the content of total reducible immature cross-links (DHLNL+HLNL+LNL), which is the biochemical collagen maturation index [21–23, 39, 40], showed no significant difference between low- and high-mineralized bone fractions in either controls ($p=0.94$) or fracture cases ($p=0.30$) (Fig. 2a).

It is thought that nonenzymatic cross-links such as Pen accumulate within collagen fibers in a time-dependent manner in senescence due to glycation and oxidation [25, 30] and that age-related accumulation of Pen results in decreased bone toughness [6]. However, we found that enzymatic cross-link content differs between fracture cases and controls (Fig. 1a). Because enzymatic and nonenzymatic cross-links, which

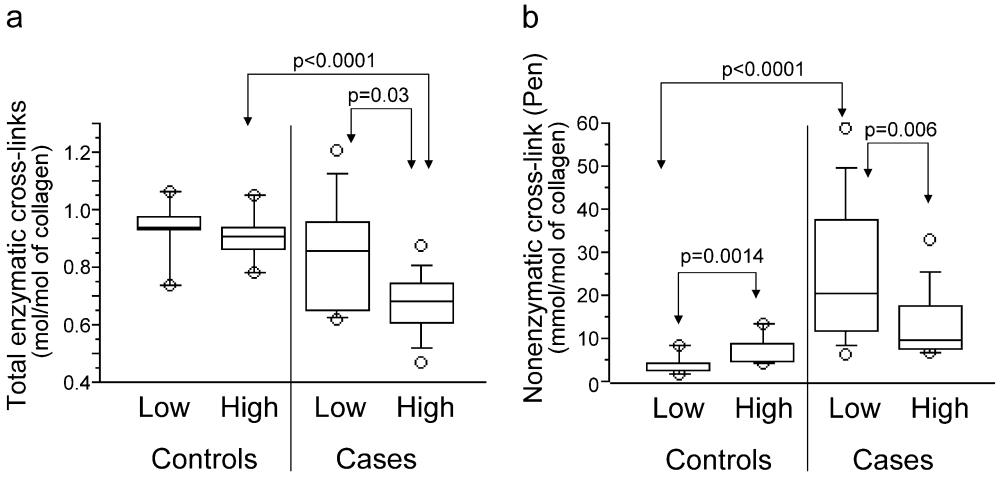

Fig. 1 Collagen cross-link contents in human femoral neck bone in cases of intracapsular hip fracture and postmortem controls. **a** Total enzymatic cross-link content (the sum of immature and mature cross-links); **b** nonenzymatic cross-link content [pentosidine(Pen)]. Data are depicted as box and whisker plots showing medians, 25th, and 75th quartiles, and complete data range

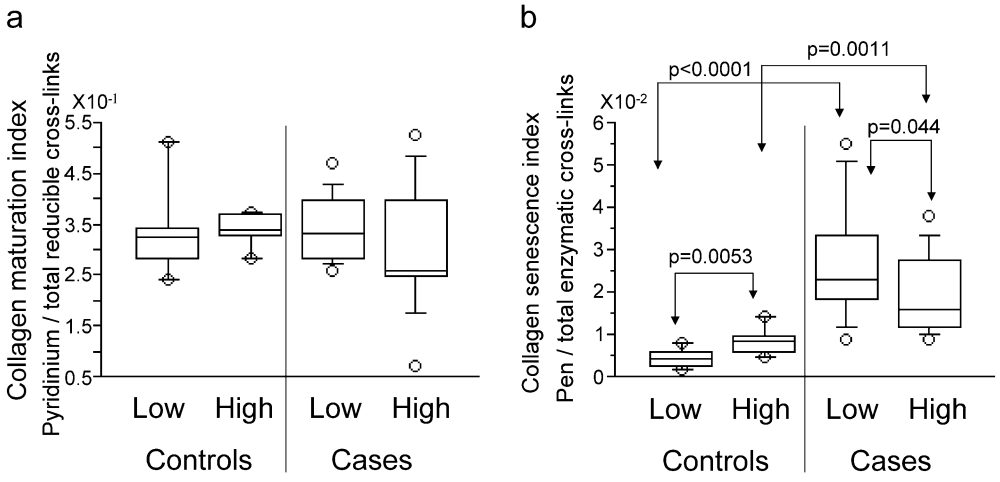

Fig. 2 Collagen maturation index (**a**) and senescence index (**b**) are estimated in human femoral neck in cases of intracapsular hip fracture and postmortem controls. **a** Collagen maturation index is the ratio of mature to immature cross-links. **b** Collagen senescence index is the ratio of nonenzymatic cross-links to total enzymatic cross-links. Data are depicted as box and whisker plots showing medians, 25th, and 75th quartiles, and complete data range

coexist in collagen fibers, have distinctive and opposing functions in bone, we propose that the biochemical index of collagen function should take both enzymatic and nonenzymatic cross-links into consideration. Thus, we calculated the ratio of the content of Pen to the content of total enzymatic cross-links (the sum of DHLNL, HLNL, LNL, Pyr, and Dpyr) and used this as the index of collagen senescence (Fig. 2b). In controls, this ratio was significantly higher in high-mineralized bone fraction than in low-mineralized bone fraction (p=0.0053) whereas for fracture cases, the ratio was significantly higher in low-mineralized bone fraction than in high-mineralized bone fraction (p=0.044) (Fig. 2b). When comparing cases within the same density fractions, this ratio for fracture cases was significantly higher than that in controls in both low- (p<0.0001) and high- (p=0.0011) mineralized bone fractions (Fig. 2b).

Lysine hydroxylation and enzymatic cross-link patterns

To compare the enzymatic cross-link pattern derived from Hyl or Lys residues in collagen between controls and fracture cases, we determined the degree of Lys hydroxylation in acid-soluble and pepsin-digestable type I collagen. Table 3 shows the degree of Lys hydroxylation and patterns of immature and mature cross-links expressed as the ratio of Hyl-derived cross-links to those of Lys-derived cross-links in bone from controls and fracture cases.

The degree of Lys hydroxylation of both pepsin-digestible collagen, which excludes the nonhelical domain of collagen, and acid-soluble collagen, which includes both nonhelical and helical domains of collagen, in bone was significantly higher in fracture cases than in controls (all comparisons, p<0.001). Solubilities of collagen from bone after acid and pepsin treatment were 3–5% and 27–32% of decalcified bone matrix, respectively. Our methods of biochemical analysis gave only an estimate of soluble fractions after acid or pepsin treatment. Therefore, we also determined the ratio of Hyl-derived cross-links to those of Lys-derived cross-links (DHLNL+HLNL/LNL and Pyr/Dpyr) (Tables 2 and 3), which are used as a parameter of tissue-specific cross-link pattern [19, 21, 22]. The higher hydroxylation of Lys in collagen from fracture case relative to controls was consistent with significantly higher values of the ratios (DHLNL+HLNL)/LNL and Pyr/Dpyr, such that enzymatic cross-link patterns correlated with the extent of Lys hydroxylation in collagen molecules (Table 3).

Table 3 Lysine hydroxylation and relative proportion of hydroxylysine-derived cross-links

	Lysine hydroxylation (%)[a]				Proportions of hydroxylysine-derived cross-links	
	Pepsin extractable collagen		Acid soluble collagen		Immature cross-links	Mature cross-links
	α1 (I)	α2 (I)	α1 (I)	α2 (I)	(DHLNL+HLNL)/LNL	Pyr/Dpyr
Controls						
Low density	18.7±1.2	20.2±2.2	20.1±1.8	21.3±0.9	8.9±0.9	2.9±0.5
High density	18.0±1.7	21.0±1.4	21.2±2.0	23.0±1.1	9.1±1.3	3.1±0.5
Fracture cases						
Low density	23.9±3.7[d]	24.4±2.2[d]	23.8±2.1[d]	23.1±1.4[c]	16.0±4.1[d]	5.9±3.6[c]
High density	22.8±2.9[d]	25.1±2.1[d]	23.7±1.7[c]	25.0±1.8[c]	12.0±3.8[b]	6.1±2.9[b]

Values are expressed as mean±SD
DHLNL dihydroxylysinonorleucine, *HLNL* hydroxylysinonorleucine, *LNL* lysinonorleucine, *Pyr* pyridinoline, *Dpyr* deoxypyridinoline
[a]Hyl/(Hyl +Lys)×100 (%)
[b,c,d]Significant differences between controls and fracture cases within the same density (low or high) at p<0.05, p<0.01, and p<0.0001, respectively

Discussion

This study is the first application of this density-fractionation method for cross-link analysis of patient sample. We also found that biochemical identification of reduction in enzymatic collagen cross-links and increase in glycation-induced Pen cross-link in both low- and high-mineralized bone in patients with hip fracture while collagen contents in bone do not differ. Further, Lys hydroxylation of collagen chains and ratio of Hyl-derived cross-links to Lys-derived cross-links were increased in patients with hip fracture.

Density fractionation and degree of mineralization

The low-density fraction is composed of less mineralized bone than the high-density fraction, suggesting that fractionation by the density gradient method of Grynpas et al. [36] reflects the degree of mineralization. Because a higher ratio of Ca/P is thought to represent a more mature crystal formation [35, 41], the high-density fraction may be comprised of not only more highly mineralized bone but also more mature crystallization than the low-density fraction. Hence, low- and high-density fractions are considered to be low- and high-mineralized bone fractions, respectively.

This is in agreement with previous reports in chick [41], rabbit [12], and porcine [35]. The degree of secondary mineralization is considered to depend on turnover rate [1, 7, 9]. We found not only that cortical bone was rich in low-mineralized bone but also that the low-mineralized bone fractions were less mineralized in fracture cases than in controls. We conclude that younger osteons are more common in fracture cases due to insufficient secondary mineralization. Such decrease in the degree of mineralization is most likely caused by elevated bone remodeling while urinary excretion of Dpyr, which is well established by bone resorption maker, was higher in fracture cases than in controls (data not shown), suggesting that bone turnover may be higher in fracture cases than in controls. Akkus et al. [42] showed that secondary mineralization of unremodeled fragments (primary lamellar bone) persisted for two decades and that these areas were replaced with secondary osteons at the fully mineralized limit. Unremodeled primary lamellar bone may have a similar degree of mineralization even at higher turnover rates such as in osteoporosis because mineralization is not affected by remodeling in primary lamellar bone. It is interesting that the high-mineralized fraction from fracture cases had similar contents of calcium and phosphorus as that of controls in spite of the fact that the high-mineralized fraction in fracture cases was smaller than that of controls. Thus, our results suggest that the low-mineralized fraction is more constrained and less mineralized in fracture cases but the high-mineralized fraction should be abundant in fully mineralized bone.

Enzymatic cross-link formation

The formation of total enzymatic cross-links in bone is strictly regulated by the action of lysyl oxidase, and excess formation cannot occur in a primary mineralization process in vitro [22, 43] or in vivo [20, 44]. Eyre et al. [24] and we [25, 45] have shown, in accord with in vitro and in vivo mineralization model analyses, that age-related changes in enzymatic cross-links reach a maximum at adolescence and subsequently stay in a similar range throughout adult life. However, significant reductions in both enzymatic immature and mature cross-links were observed to a similar extent in fracture cases without a change in collagen maturity (Figs. 1a, 2a). This result indicates that the initial step of cross-linking may be inhibited without affecting the conversion of immature cross-links to mature forms. These results are in accord with observations made by Bailey et al. [46] and Oxlund et al. [14] on collagen of cancellous bone from the femoral head and neck of osteoporotic patients. They found a 20–44% reduction of enzymatic cross-links in osteoporotic cancellous bone.

The first step of enzymatic cross-link formation is oxidative deamination of Σ-amino groups of Lys and Hyl residues in telopeptides [20, 47]. Thus, the previous reports [14, 46] and our data indicate that this first step is hampered by a negative regulator of lysyl oxidase. The mechanism underlying the inhibition of collagen cross-linking in osteoporotic bone is not known. Candidates for the inhibitors of lysyl oxidase have been proposed. Reynolds et al. [48] have shown that hip fracture patients may be deficient in vitamin B6. Vitamin B6 is an essential coenzyme for lysyl oxidase [49]. In an earlier study, we demonstrated that vitamin B6 deficiency in the normal rat led to impaired enzymatic immature reducible cross-link formation [50]. Masse et al. [51] found that vitamin B6 deficiency in chick resulted in reduction of bone strength. Thus, latent vitamin B6 deficiency may account for impaired enzymatic cross-link formation and subsequent reduced bone strength in osteoporosis.

There is recent evidence that a mildly elevated plasma level of homocysteine in the general population is a common condition, and this mild hyperhomocysteinemia may be a risk factor for hip fracture independent of a reduction of bone mineral density [52, 53]. Hyperhomocysteinemia may have a detrimental effect on bone strength. The underlying pathophysiological mechanism for the occurrence of hip fracture in patients who have moderately elevated plasma homocysteine is not understood. Because homocysteine is known to inhibit lysyl oxidase, a hypothesis was proposed that mildly elevated plasma homocysteine in the general population may involve a disturbance in collagen enzymatic cross-link formation in bone [54, 56]. Further study is needed to elucidate the correlation between lysyl oxidase regulators and actual enzymatic cross-link formation in human bone. An excessive accumulation of AGEs may also be due to the reduced contents of enzymatic cross-links formed in fracture cases. AGEs are thought to be formed between sugars and Lys residues, and Lys residues are one of the

several essential sites of enzymatic cross-linking in collagen. Thus, formation of AGEs results in competitive inhibition of enzymatic cross-link formation [57].

Nonenzymatic cross-link formation

Biochemical data reported here comprise the first evidence from normal human subjects that AGEs are accumulated more in high-mineralized bone, which consists of older osteons, than in low-mineralized bone, which consists of younger osteons. It is reasonable to assume that Pen accumulates in high-mineralized older bone in controls because nonenzymatic glycation [17, 58] or oxidation [30] occurs spontaneously with tissue maturation and subsequent senescence. Surprisingly, Pen content was greater in low-mineralized bone than high-mineralized bone in fracture cases despite the fact that low-mineralized bone consists predominantly of younger osteons. The mechanism of acceleration of nonenzymatic cross-link formation fracture cases is not known. Pen consists of single Lys and arginine moieties cross-linked by pentose [31]. However, the major in vivo carbohydrate source leading to Pen formation is not known. What is clear is that oxidation reactions are required at some stage in the formation of Pen [59]. Therefore, Pen is considered a glycoxidation product [59]. Because fracture cases did not show hyperglycemia in this study, the high accumulation of AGEs and Pen may be predominantly due to increased oxidation reactions rather than glycation-induced by hyperglycemia.

Collagen maturity and senescence

Earlier studies support the evidence that under physiological conditions enzymatic cross-link formation and conversion of immature to mature cross-links reach a maximum during the primary mineralization process [20, 22, 39, 40, 43, 44]. Because low- and high-mineralized bone fractions are considered not to include both the osteoid-rich and extremely young bone in bone forming surfaces [34, 35], collagen maturation of low- and high-mineralized fractions may reach a maximum. Actually, the collagen maturation index estimated by the ratio of enzymatic cross-links (Fig. 2a) did not differ between fractions, suggesting that enzymatic cross-link maturation is not impaired. Thus, there is a limitation to merely determining the ratio of enzymatic cross-links for estimation of maturity or senescence of matrix in the deeper zone. Senescence may occur in the deeper zone after maturation. Moreover, excessive formation of Pen in bone is thought to deteriorate the mechanical properties of bone, particularly postyield properties and toughness, consequently making collagen fibers brittle [6]. In fact, the brittle matrix has been reported to rely considerably on microcrack formation in bone [17]. On the other hand, Oxlund et al. [15] demonstrated in an animal study that impaired enzymatic cross-link formation in bone by the treatment of beta-aminopropionitrile, which irreversibly inhibits the enzyme lysyl oxidase, results in significant decrease in bone strength down to approximately 30% of control animals. Thus, enzymatic crosslinking has beneficial effects on proper bone strength without excessive brittleness. Thus, we propose that the index for the assessment of senescence and fragility of collagen should take both enzymatic and nonenzymatic cross-link formation into consideration (Fig. 2b) while enzymatic and glycation-induced cross-links have opposing functions in bone [6, 15]. Our data showed that this index of collagen senescence was significantly higher in high-mineralized bone consisting of older osteons than in low-mineralized bone consisting of younger osteons in controls. Thus, both the collagen maturity index and collagen senescence assessment may be valuable tools for estimation of collagen quality in bone.

Lys hydroxylation

The increased degree of Lys hydroxylation in bone from he fracture cases reported here is in accord with previously reported studies in human osteoporotic bone [46]. Furthermore, we measured Lys hydroxylation and the ratio of Hyl-derived cross-links to Lys-derived cross-links (Table 3). The increased hydroxylation of Lys in fracture cases was associated with an increase in that ratio, indicating that the specific Lys residues involved in cross-link formation are converted to Hyl. Posttranslational hydroxylation of Lys residues in collagen varies in calcified and noncalcified tissue and with stages of development [20, 22]. Such different degrees of Lys hydroxylation between tissues or in the distinctive differentiation stages is regulated by the three types of Lys hydroxylases [19, 21, 27]. However, the mechanism underlying overhydroxylation of Lys in osteoporotic bone collagen is not understood because little is known about the regulatory factors in the expression of Lys hydroxylases. Recently, Pornprasertsuk et al. [29] demonstrated that overhydroxylation of Lys residues induced by overexpression of Lys hydroxylase 2b in a MC3T3-E1 osteoblastic cell culture resulted in delayed mineralization and reduced diameter of collagen fibrils. Thus, there is a critical role for Lys hydroxylation and catalyzed cross-linking in collagen matrix formation and mineralization in bone. It is postulated, therefore, that both quantitative changes in enzymatic cross-links and the cross-link pattern based on the degree of Lys hydroxylation are responsible for qualitative features of collagen in bone.

Limitations exist for interpretation of results of this study. First, there is no information on relationship between bone mechanical properties and biochemical parameters such as collagen and mineral phase. While the bone specimen from patients was quite small, we could not obtain sufficient bone fragment for the mechanical test. Thus, further work will be required to evaluate the correlation between mechanical properties and not only collagen quality and mineral phase but also microdamage accumulation. The second, Pen, is a glycation or oxidation-induced cross-link and the only detectable advanced glycation end products. Other unknown glycation-induced

cross-links may exist in bone and have a similar biological effect on collagen mechanical properties. Thus, Pen content in bone collagen is considered a marker to estimate other glycation-induced cross-linking. However, it is not clear that Pen content in bone is proportional to the other glycation-induced cross-linking. Therefore, quantitative determination of Pen may be only a rough estimation of the detrimental effect of glycation-induced cross-links.

In conclusion, the results of this study indicate that the relation between impaired secondary mineralization of human bone and its related alteration of collagen posttranslational modifications implies that bone quality measures, as determined from bone composition, could be important for explaining the variation of fracture susceptibility in individuals, independent of alteration of bone mass.

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