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A Comparative Study of the Distribution of the Stable Crosslink, Pyridinoline, in Bone Collagens from Normal, Osteoblastoma, and Vitamin D-Deficient Chicks

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Summary

Tryptic peptides of bone collagens from 4-week-old normal, osteoblastoma and vitamin D-deficient chicks were studied using gel filtration chromatography. Absorbance at 230 nm and fluorescence (excitation at 330 nm, emission at 390 nm) of each fraction were measured. The relative quantities of each peak from the absorbance and fluorescence patterns were semiquantified by planimetry. Osteoblastoma bone collagen had a prominent, fluorescent, crosslinked peptide that contained pyridinoline. Fluorescence of this pyridinoline-containing peak in AO collagen was much greater than in the vitamin D-deficient and normal bone collagen counterparts. A comparison of fluorescence patterns clearly showed that the distribution of pyridinoline in collagen from normal and diseased bone was totally dissimilar.

The dissimilarities in distribution of pyridinoline in these bone collagens may be attributed to differences in the degree of lysine hydroxylation, to the degree of mineralization, or some other factor.

Introduction

Most crosslinking amino acids have been detected only after reduction with [^{3}H]-$NaBH_4$; however, two new crosslinking amino acids have been identified without prior chemical reduction (nonreducible crosslinks) (7,10). One of these stable, nonreducible crosslinks is pyridinoline, a trifunctional, fluorescent amino acid present in tendon, bone, cartilage, and hypertrophic scar. However, nothing is known about the distribution of pyridinoline in collagen under normal or pathological conditions. This compound is reputed to

Abbreviations

DHLNL	dihydroxylysinonorleucine
HLNL	hydroxylysinonorleucine
N	Normal
AO	avian osteogenic osteoblastoma
D-	vitamin D-deficient
λ_{EX}	excitation wavelength
λ_{EM}	emission wavelength

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play a significant role in collagen maturation (6). In the latter context, maturation denotes the transition from collagen that contains reducible, nonstable, crosslinks, to collagen that contains both nonstable and stable crosslinks.

The latter transition is a continuing process in a time frame of months or years, but stable crosslinks can be detected in chick bone within weeks after hatching. The state of maturation of collagen molecules involves chemical reactions that can be independent of biological age. Collagen maturation may be affected by the degree of lysine hydroxylation, collagen organization, aldehyde formation, mineralization or other factors (5,8,14).

Bones from both osteoblastoma and vitamin D-deficient chicks are hypomineralized at 4 weeks after hatching, exhibit high ratios of reducible crosslinks (DHLNL/HLNL) characteristic of newly synthetized collagen and have large amounts of osteoid (1,2,4,12,13,14). Yet bones from both these diseased chicks contain a considerable quantity of the stable crosslink pyridinoline.

As a first step towards understanding bone collagen maturation under normal and pathological conditions, we have studied the distribution of pyridinoline in tryptic peptides from three different kinds of bone collagen (normal, AO, and vitamin D-deficient) using Sephadex G-50 Superfine gel chromatography.

Materials and Methods

Virus--An endpoint-purified derivative of avian myeloblastosis virus (MAV-2(0)) was used. The virus was propagated by serial passage in chickens. Blood was collected from birds manifesting heavy osteoblastoma (palpably thickened long bones), and the sera were prepared, pooled and stored at -70°C in 0.1 ml portions (16).

Experimental animals--White Leghorn chicken eggs were used in all experiments (SC strain, Hy-line International, Dallas Center, Iowa). MAV-2(0) ($2-3 \times 10^4$ PFU/0.1 ml phosphate buffered saline pH 7.4, containing 10% calf serum) was administered to 12-day-old embryos by injection of a vein in the chorioallantoic membrane. All eggs were maintained in a humidified incubator until hatching; infected and control eggs were transferred to separate hatchers. After hatching, birds were maintained in separate brooders. Normal and osteoblastoma chicks were fed Purina Growena chick diet and water *ad libitum*. Vitamin D-deficient chicks were fed a Teklad diet containing 1.4% calcium, 1.1% phosphate and no vitamin D (Teklad Test Diets, TD79342, Madison, Wisconsin).

Preparation of collagen from bone--Clean tibia diaphyses from control and experimental animals were frozen in liquid nitrogen and reduced to powder using a Spex Freezer Mill (Spex, Inc., Metuchen, New Jersey). Bone powders were demineralized by 4-5 successive 24 h treatments with 0.5M EDTA pH 7.4 at 4°C with constant stirring. Samples were washed with 0.02M NH_4HCO_3, then distilled H_2O, and lyophilized.

Trypsin digestion of collagen--The collagens were suspended and stirred constantly in 0.05M Tris-HCl, pH 8.0, 1 mM $CaCl_2$ and denatured for 20 min by

heating at 65°C. The suspension was cooled to 37°C and digested with trypsin (1% W/W) (TPCK-trypsin, Cat. #30C637, Millipore Corp., Freehold, New Jersey) for 4 hr at 37°C. The mixture was heated to 60°C for 15 minutes. The collagen was almost completely solubilized (98%). The solution was cooled to 37°C and treated with trypsin (0.5% W/W) for 2 h with constant stirring, then lyophilized (Y. Kuboki's modification of (3)).

Chromatographic methods--Gel filtration chromatography was carried out on a column of Sephadex G-50 Superfine (1.9 x 230 cm) equilibrated with 2M guanidine-HCl (Pierce Chemical Co., Sequanal grade, Rockford, Illinois), 0.05M Tris-HCl, pH 7.5. Portions of dried tryptic peptides were dissolved in 5M guanidine-HCl, 0.05M Tris, pH 7.5, and denatured for 20 min. at 50°C then applied to the column after a small insoluble residue (1-2%) was removed by centrifugation (40 min., 25,000g). Fractions (5 ml) were collected and the absorbance at 230 nm measured. The fluorescence of each fraction was measured using a Hitachi fluorescence spectrophotometer, with excitation at 330 nm and emission at 390 nm. Peak quantities from absorbance and fluorescence patterns were determined by planimetry.

Reduction--Some portions of bone powder were reduced with [^{3}H]-$NaBH_4$ (100 mCi/mmole) as described before (11).

Results

The figure shown here represents chromatograms of gel filtration (Sephadex G-50 Superfine) of tryptic peptides from normal, osteoblastoma, and vitamin D-deficient bone collagens. In absorbance patterns (solid line) the most pronounced difference among these three chromatograms was the amount of peak I material (near the exclusion volume of the column), though the overall patterns were almost identical.

The amount of peak I relative to the amount of total absorbance was 22.6% in vitamin D-deficient, 14.9% in normal and 9.1% in AO. The absorbance ratios of peak I to peak III were 11 in vitamin D-deficient, 8.0 in normal and 2.6 in AO (Table 1).

The fluorescence patterns of the three groups were markedly different. AO bone collagen contained a prominent fluorescence peak designated peak III (Figure 1). The exitation and fluorescence spectra of this fraction, as well as other fluorescent peaks, were measured and showed λ_{EX} max at 330 nm and λ_{EM} max at 390 nm. The latter values are consistent with the established excitation and emission properties of peptidyl pyridinoline in neutral conditions (7, 9). Moreover, the presence of pyridinoline in these peaks was confirmed by Dr. David Eyre using a Glenco amino acid analyzer programmed to separate pyridinoline from other amino acids. In addition, hydroxyproline was identified in these fractions after amino acid analysis of acid hydrolyzates, indicating that these were collagen peptides. The fluorescence ratios of peak I to peak III were 4.4 (in N), 0.20 (AO), and 1.4 (D-). Moreover, the ratio of

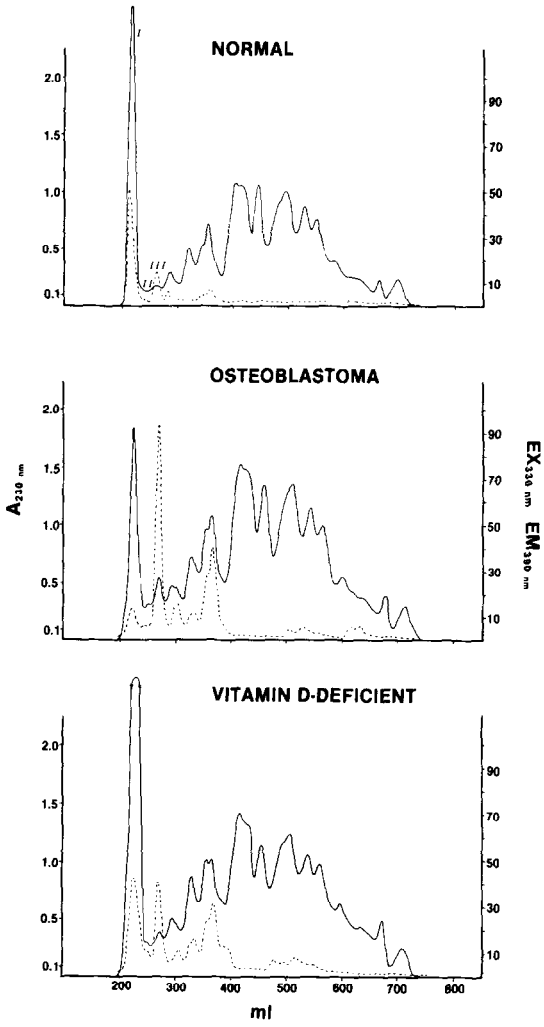

Figure 1

The chromatograms in Figure 1 represent separations of tryptic peptides of collagen (ca. 120 mg samples) on a Sephadex G-50 superfine column (1.9 x 230 cm) eluted with 2.0M guanidine HCl, 0.05M Tris, pH 7.5. The UV absorbance at 230 nm _____, and fluorescence emission at 390 nm with excitation at 330 nm ----- were measured. Samples were obtained from tibiae of 4-week-old White Leghorn chicks, normal, osteoblastoma (embryos infected on day 11 in ovo with myeloblastosis-associated virus-2(osteo)), and vitamin D-deficient (chicks fed a rachitogenic diet that lacked vitamin D). Peak I is near the excluded volume of the column; II, a peptide present in slight quantity in osteoblastoma and vitamin D-deficient but not normal bone collagen; III, a major fluorescent, pyridinoline-containing peak found in abundance in collagen from osteoblastoma and vitamin D-deficient bone but not normal bone.

fluorescence to absorbance for peaks I and III dramatically illustrated the relative amounts of pyridinoline in D- and AO collagen (Table 1). Moreover, total fluorescence of collagen from AO or vitamin D-deficient chicks was greater than that for normal bone collagen (Table 1).

Table 1

Ratios[1] of Absorbance and Fluorescence

	I/III[2]		Total[3]	EM_{390}/A_{230}[4]	
	A_{230} nm	λ_{EM} 390 nm	EM_{390}/A_{230}	I	III
N	8.0	4.4	0.08	0.36	0.65
AO	2.6	0.20	0.17	0.15	2.5
D-	11	1.4	0.14	0.17	1.3

[1]Absorbance at 230 nm and fluorescence emission at 390 nm (EX_{330} nm) were measured for tryptic peptides of collagen from 4-week-old bones from normal (N), osteoblastoma (AO) or vitamin D-deficient (D-) chicks.

[2]Approximately 18 absorbance peaks and 5-6 fluorescence peaks (in the first half of the chromatogram) were detected for each collagen sample separated by G-50 Superfine gel chromatography. The value represents the ratio of planimetry units for peak I (void volume) to peak III (a major, pyridinoline-containing, crosslink peptide).

[3]Ratio of total fluorescence to total absorbance for N, AO, and D- collagen (120 mg) from G-50 superfine chromatography.

[4]Ratio of fluorescence emission to absorbance for peaks I and III.

Fluorescence in tryptic peptides of blood and plasma from AO chicks was analyzed to discern if these two contaminating components in bone contained fluorescence in the peak III region of the chromatogram. No fluorescence was detected in peptides from either plasma or blood; however, the peak I region of plasma contained some fluorescent material (data not shown).

In addition, the radioactivity of each fraction was quantitated using the same bone collagen after reduction with $[^3H]NaBH_4$. This analysis (data not shown) indicated a great deal of radioactivity present in peak I but background levels in peak III in all cases. The latter information indicates that only low quantities of precursor aldehyde or reducible crosslinks are present in peak III. Identical results have been obtained for replicate experiments performed using two or three different chicks from each group.

Discussion

The findings reported here demonstrate that the nonreducible, stable crosslinking amino acid, pyridinoline, exists in bone collagen from

osteoblastoma chicks, as well as in collagens from vitamin D-deficient and normal chicks. However, the distribution of pyridinoline in collagen peptides is unique for each case. Moreover, pyridinoline exists in osteoblastoma and vitamin D-deficient bone collagens in greater quantity than in the normal counterpart. In particular, osteoblastoma collagen has less very highly crosslinked collagen than does normal or vitamin D-deficient bone collagen, as indicated by the relative sizes of peak I (Peak I, Figure 1, AO < N < D-). These collagens contain a peak, common to both (Peak III, Figure 1), as well as other pyridinoline-containing peaks, not present in abundance in normal collagen. Data in Table I indicate that this peptide (Peak III) is most abundant in osteoblastoma, relative to A_{230} absorbing material (Table 1).

The profiles of reducible crosslinks of osteoblastoma or vitamin D-deficient bone are characteristic of immature collagen (high dihydroxylysinonorleucine/hydroxylysinonorleucine ratio) (2,14). However, collagens from the osteoblastoma and vitamin D-deficient bone contain an appreciable quantity of the stable crosslink pyridinoline, considered characteristic of mature collagen. How does one reconcile the presence of a stable crosslink in immature collagen? It has been reported that excessive hydroxylation of lysine residues in collagen results in the formation of an abundance of hydroxylysine-derived crosslinks, including the reducible crosslinks dihydroxylysinonorleucine (DHLNL), and hydroxylysinonorleucine (HLNL) (5). It has been proposed that DHLNL may participate as an intermediate in pyridinoline formation (6,9). Our results are consistent with this thought, because in osteoblastoma and vitamin D-deficient bone collagen, hydroxylation of lysine residues is increased (N, 23%; AO, 36%; D-, 29%) and results in an increased ratio of DHLNL to HLNL (2,14, and Banes and Mechanic, unpublished results). Another characteristic of both diseases is hypomineralization of bone. Therefore, it is possible that in both disease states, there is a compartment of nonmineralized collagen that matures from nonstable to stable crosslinks. The lack of complete mineralization of collagen in AO and D- bone may permit rapid transition of reducible, nonstable, to nonreducible, pyridinoline crosslinks.

Therefore, excessive collagen lysine hydroxylation leading to increased quantity of precursor (DHLNL) to pyridinoline, and/or accelerated transition from reducible to pyridinoline crosslinks may explain the presence and abundance of pyridinoline in bone collagen from AO and D- chicks. The differences in distribution of pyridinoline in the tryptic collagen peptides could reflect excess hydroxylation of lysine and subsequent pyridinoline formation at different sites in the molecules.

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