Arch Jpn Chir 50(6), 789~795, Nov., 1981

In Vitro Aging of Pyridinoline Crosslinks in Bone and Tendon Collagens

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Received for Publication, Sept. 9, 1981.

Introduction

A unique feature of collagen is that it increases in mechanical and chemical stability with age in vivo. These changes may be attributed to a gradual increase in the number of intermolecular crosslinks and/or in chemical stability. Most of the known crosslinks of collagen are aldimines or related structures isolated after chemical reduction with $NaBH_4$[11]. The number of these crosslinks[11] as well as lysyl oxidase activity[10], which catalyses the first and the only enzyme dependent step in the crosslinking process of collagen, gradually decreases with age, so the aging change of collagen is not explained by the stabilization of these crosslinks. They are, therefore, considered as intermediate substances to produce mature and stable crosslinks[2,4].

These aging changes of collagen also appear to take place during aging in vitro, although at an accelerated rate. Collagen that is incubated at 37°C gradually loses solubility, and the number of reducible crosslinks decreases concomittently[9].

Recently a fluorescent crosslinking amino acid was isolated from collagen of ox tendon and bone and named pyridinoline[5]. Pyridinoline gradually increases with age in several tissues[8] and from its chemical structure it is thought to be formed by spontaneous reaction from the reducible crosslink DHLNL[3]. Pyridinoline is possibly one of the long anticipated "mature" non-reducible crosslinks of collagen.

This paper describes the changes in amount of pyridinoline in collagen of bone and tendon during aging in vitro.

Materials and methods

Preparation of collagens: Cortical bone of adult rabbits was pulverized in liquid nitrogen and decalcified by repeated extraction with 0.5 M EDTA at pH 7.4 and then extracted with 0.5 M acetic acid. The insoluble collagen was washed with distilled water and lyophilized. Bovine bone collagen was obtained from the cortical bone of 3-year-old cows, and insoluble collagen was

Abbreviation: DHLNL, Dihydroxylysinonorleucine.
Key words: Collagen, Pyridinoline, Crosslink, Aging, In vitro.
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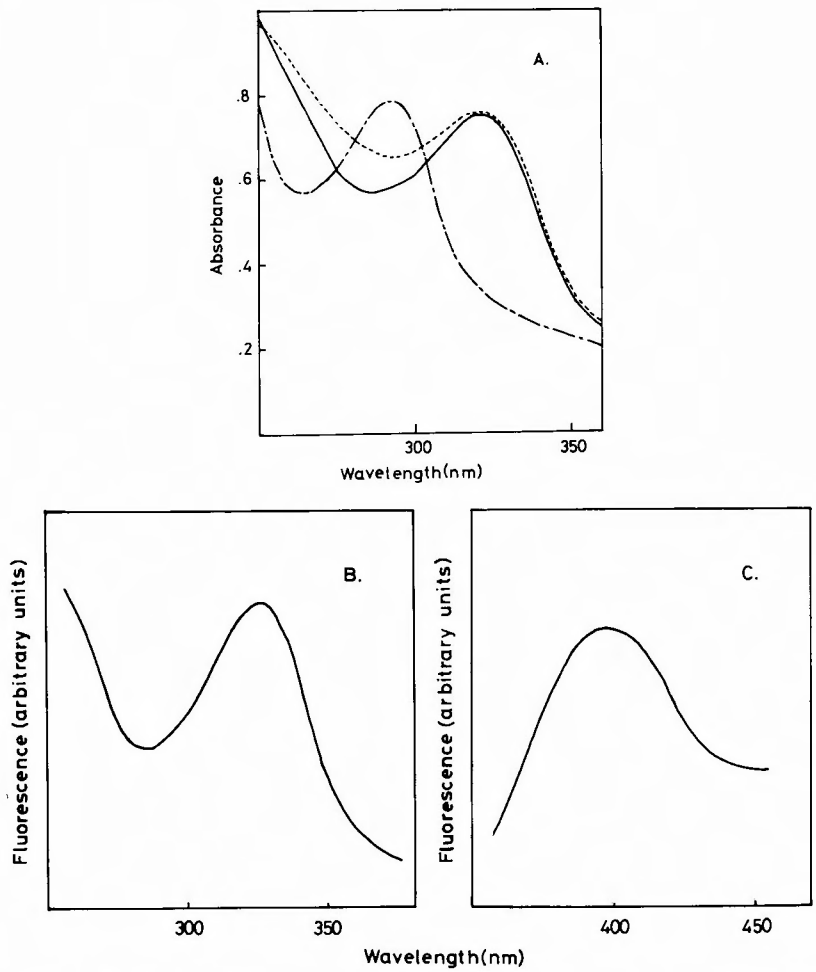

Fig. 1. *U.v.-absorption and fluorescence-activation and -emission spectra of purified pyridinoline.* (A) U.v.-absorption spectrum in 0.1 N-HCl (—·—·), in 0.02 M-sodium phosphate buffer, pH 7.4 (----), and in 0.1 N-NaOH (——). (B) Activation spectrum in 0.02 M-sodium phosphate buffer, pH 7.4; emission was monitored at 400 nm. (C) Fluorescence spectrum in 0.02 M-sodium phosphate buffer, pH 7.4; excitation was at 325 nm.

obtained by the same method as rabbit bone collagen. Bovine tendon collagen was obtained from Sigma Chemical Co., St. Louis. Freshly prepared bovine tendon insoluble collagen was also obtained from 3-year-old cows by successive salt and acid extraction.

Aging in vitro of collagens: Samples containing 40–50 mg of collagen were incubated in triplicate or quadruplicate without continuous shaking for various periods of time at 37°C in 0.15 M NaCl 0.05 M Tris-HCl buffer, pH 7.4. Some samples were incubated at 4°C under the same conditions except for temperature. After incubation for a prescribed period, they were washed with cold water and lyophilized.

Analytical procedures: Acid hydrolysis was carried out with 6N HCl in sealed tubes under nitrogen at 110°C for 24 hr. The HCl was removed by evaporation in vacuo at 60°C. Pyridino-

line was separated from the acid hydrolysate using P-cellulose chromatography as described by FUJIMOTO and MORIGUCHI[6]. The collected fractions had fluorescence and u.v.-absorption spectra (Fig. 1) identical with those previously reported[5]. The pyridinoline content was determined by fluorescence assay (excitation 295 nm, emission 395 nm) after P-cellulose chromatography using JASCO FP-550 fluorescence spectrophotometer[6]. The hydroxyproline content of the same hydrolysate was determined by WOESSNER's method[12] and the pyridinoline content of collagen was expressed as the number of residues of pyridinoline/100 residues of hydroxyproline.

Results and discussion

The amino acid composition of rabbit bone and bovine tendon insoluble collagens are shown in Table 1. They show the typical amino acid composition of type I collagen. The amino acid composition, including Hyl content, did not change during in vitro aging.

The pyridinoline content of both rabbit and bovine bone collagens increased significantly during in vitro aging (Fig. 2A, B). Inhibition of the increase of pyridinoline during in vitro aging of bovine bone collagen at 4°C is consistent with the fact that in vitro aging observed as the function of the content of aldimine crosslinks is also inhibited at 4°C (Fig. 2B)[9].

Table 1. Amino acid composition of rabbit bone and bovine tendon collagen

	Rabbit bone		Bovine tendon	
		after 4 wk*		after 4 wk*
Hyp	107.5	112.4	111.5	113.7
Asp	48.6	44.0	46.2	45.2
Thr	21.0	19.4	17.3	17.6
Ser	33.0	31.8	33.3	33.4
Glu	73.8	71.2	72.1	71.4
Pro	104.7	110.7	116.9	121.5
Gly	324.9	330.7	321.8	316.0
Ala	109.5	105.4	102.7	101.1
Cys	nd	nd	nd	nd
Val	26.4	26.1	18.1	25.6
Met	8.1	8.9	5.9	6.1
Ile	11.5	11.0	12.7	12.9
Leu	26.9	25.2	28.4	27.6
Tyr	3.8	5.5	5.1	4.9
Phe	13.2	14.8	15.7	15.8
Hyl	8.9	8.9	14.1	13.7
Lys	27.7	25.1	20.3	21.0
His	4.5	5.5	7.5	4.4
Arg	46.0	43.4	50.4	48.1
	1000.0	1000.0	1000.0	1000.0

* Amino acid composition of collagen incubated at 37°C for 4 weeks
nd: not detectable

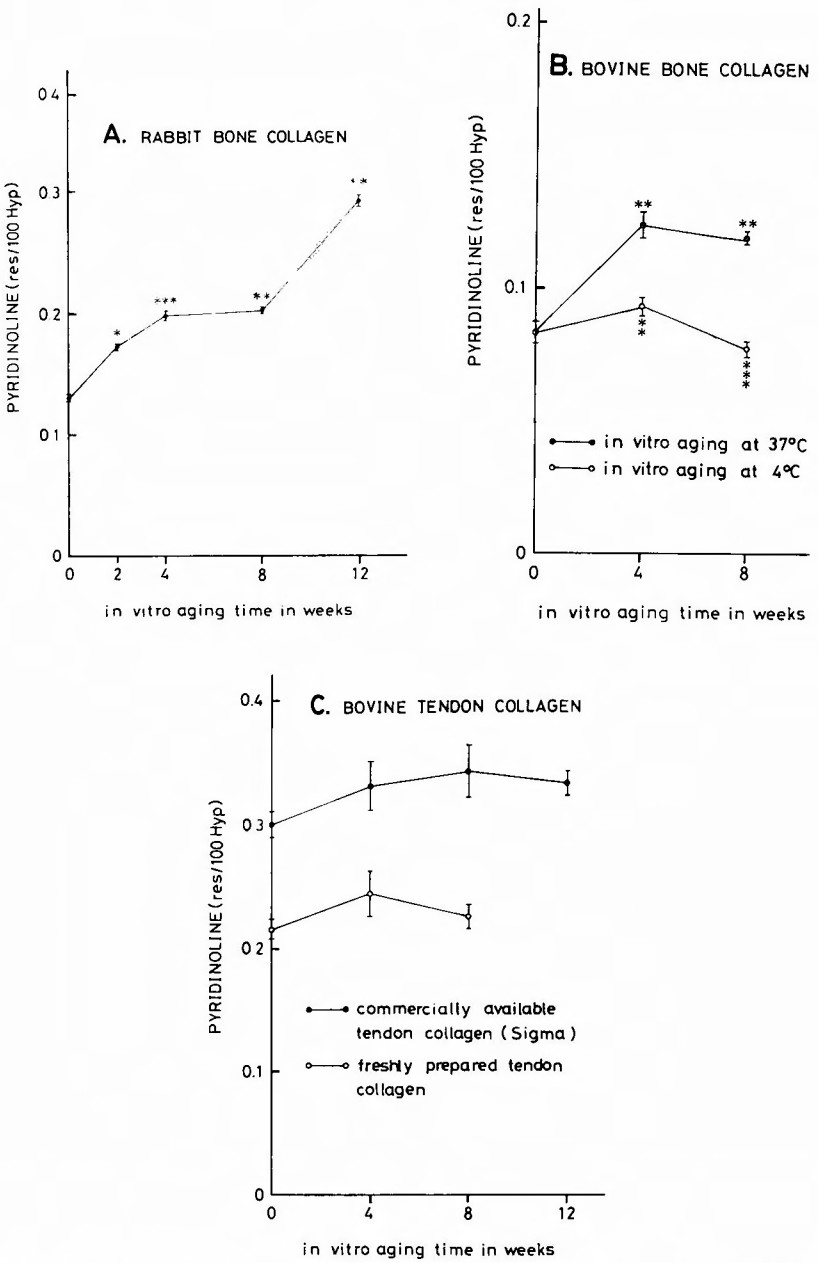

Fig. 2. *Changes of pyridinoline content during in vitro aging of collagen.* The pyridinoline content of rabbit bone collagen (A) and bovine bone collagen (B) increased significantly with age in vitro compared with the value at 0 week (*: $P< 0.05$, **: $P< 0.01$, ***: $P< 0.001$). The increase of pyridinoline was inhibited when the collagen was incubated at 4°C. The pyridinoline content at 4°C was significantly lower than the 37°C counterpart ($\overset{*}{*}$: $P< 0.01$, $\overset{*}{*}$: $P< 0.001$) (B). Bovine tendon collagen showed no increase of pyridinoline during in vitro aging (C).

On the other hand, neither commercially available nor freshly prepared bovine tendon collagen showed an increase of pyridinoline during in vitro aging (Fig. 2C). The possibility that commercially available tendon collagen had already "aged" in air was ruled out by the result of freshly prepared tendon collagen. The difference of pyridinoline content between the two tendon collagens may be attributed to the different ages of the animals (Fig. 2C).

Pyridinoline is thought to be formed by non-enzymatic conversion from the reducible crosslink DHLNL[3]. Although bone collagen is known to have one major reducible crosslink, DHLNL, tendon collagen also contains a sufficient amount of DHLNL[1], which could be converted to pyridinoline during in vitro aging.

Although both are type I collagen, bone and tendon collagens have very different physical characteristics, such as swelling properties and shrinkage temperature[7]. Some difference in assembly of the collagen molecules may be working against de novo formation of pyridinoline in tendon collagen during in vitro aging. As the tendon collagen which was extracted with EDTA did not show increase of pyridinoline content during in vitro aging (data not shown), the increase of pyridinoline in bone collagen during in vitro aging could not be attributed to the process of decalcification. Our results suggest that an increase of pyridinoline content is responsible for the increase of stability of bone collagen with age.

Summary

During incubation of rabbit and bovine bone insoluble collagen with physiological buffer at 37°C, the content of pyridinoline, non-reducible crosslink of collagen, increased significantly. The increase of pyridinoline content did not occur when bovine bone collagen was incubated at 4°C. Bovine tendon collagen, on the other hand, showed no increase of pyridinoline content during incubation.

Acknowledgements

Gratitude is extended to Drs. K. YASUHIRA, M. IGARASHI, K. TOMITA and D. FUJIMOTO and to Mrs. K. KOGISHI for pertinent advice and technical assistance and to Dr. A. CARY for critical reading of the manuscript.

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After the preparation of this paper, the similar work of Dr. D. FUJIMOTO came to our attention, when we read the abstract of his forthcoming presentation at the thirteenth annual meeting of the Japanese Society of Connective Tissue Research to be held in Tokyo on August 1–2, 1981, which was published in a supplement of the official journal of the society. Our results seem to confirm his and vice versa, although the research was done completely independently.

和文抄録

骨・腱コラーゲン，ピリジノリン架橋の in vitro aging について

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コラーゲンは動物の加齢に伴ない化学的安定性を増す．これまでに知られている環元性架橋は動物の加齢と共に減少し，加齢に伴なう安定性の増加に相応しない．そこで環元性架橋は，時間と共にさらに安定な架橋物質に変化してゆく中間物質であると考えられている．最近報告された非還元性架橋物質であるピリジノリンは，いくつかの組織で加齢に伴なって増加することが観察されており，安定な最終的架橋物質の１つである可能性がある．一方 in vivo におけるコラーゲンの加齢変化とよく似た現象が，コラーゲンを in vitro の系にうつした場合にも観察される．すなわちコラーゲンを 37°C の緩衝液中に入れておくと，時間の経過と共に，不溶化がおこり，同時に環元性架橋は減少する．そしてこの変化は 4°C では抑制される．この現象はコラーゲンの in vitro aging として知られているが，ピリジノリンが，環元性架橋から生成される最終架橋物質であるなら，in vitro aging の系においても時間と共にこの物質の増加が起ることが予想される．

そこで今回，牛及び家兎の骨コラーゲン，牛アキレス腱コラーゲンを用い in vitro aging に伴なうピリジノリン量の変化を検討した．その結果は以下のごとくである．

1) 家兎骨及び牛骨コラーゲン中のピリジノリン量は 37°C における in vitro aging に伴ない有意に増加した．
2) 家兎骨コラーゲンでは，ピリジノリン量の in vitro aging に伴なう変化は 4°C において有意に抑制された．
3) アミノ酸組成，特に Hydroxylysine 量は in vitro aging による変化が見られなかった．
4) 骨コラーゲンと異なり，牛アキレス腱コラーゲンでは in vitro aging によるピリジノリン量の変化は見られなかった．

以上の結果から，骨では加齢に伴なうコラーゲンの安定性の増加が，ピリジノリン量の増加によって起っていることを示唆すると考える．