AGE-RELATED CHANGES IN THE REDUCIBLE CROSSLINKS OF HUMAN TENDON COLLAGEN

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Received 20 May 1974

1. Introduction

A correlation has been made between the increasing age of animals and the progressive increase of collagen fibre stability when subjected to physicochemical tests, e.g. thermal denaturation, swelling and solubilization with various solvents [1–4]. These altered properties during collagen maturation may possibly be related to the total number and chemical nature of the covalent intermolecular crosslinks of collagen [5]. Some studies have shown that there are changes in the relative abundance of the borohydride-reducible crosslinks with increasing age of experimental animals, and the results seem to vary with species and tissue [6–8].

Aging is of special interest in man, both because of substantial longevity compared to most other animals, and because of the role that diseases and degenerative changes may play in human collagenous tissues. In this report we describe the alterations in crosslinking of normal human tendon collagen as a function of each decade of age.

2. Materials and methods

The deep palmar flexor tendons of Japanese citizens ranging from 2 to 74 years of age were dissected within 48 hr after accidental death. The tendons were sequentially extracted with cold 1 M NaCl and 0.1 M acetic acid and were then frozen in liquid nitrogen, powdered by crushing, and lyophilized. The dry powder was suspended in 500 vol (v/w) of sodium phosphate buffer, pH 7.5, ionic strength 0.16 and was hydrated at 5°C for one week. The samples were transferred to a 37°C water bath and a drop of Antifoam B (Dow Corning) was mixed in, followed by rapid addition of a 100-fold molar excess of NaB^3H_4 (approximately 50 mCi per mmole) [9]. Following incubation at 37°C for 30 min the protein suspension was filtered on miracloth, washed exhaustively with distilled water and then lyophilized. The tritiated proteins were hydrolyzed in 3 M HCl by refluxing for 48 hr, and the hydrolysate was evaporated [10].

A portion of each hydrolysate was measured for specific radioactivity. The data were expressed both in terms of the original weight of labeled sample (weighed prior to hydrolysis) and in terms of the measured hydroxyproline content of the hydrolysate [11]. Chromatographic fractionation of the radioactive components in each hydrolysate was carried out by using an amino acid analyzer as previously described [10]. Radioactivity was monitored in a toluene based scintillation fluid employing Beckman Biosolve-3.

3. Results and discussion

The changes in specific radioactivity of borohydride-reduced human flexor tendon with age are shown in table 1. These results show a progressive decrease in specific radioactivity with advancing age, with the oldest sample incorporating only 20% of the tritium taken up by the youngest sample. Fig. 1 shows that there are marked differences in the elution profiles of four representative samples. In all of the profiles two unknown components (K,L) appear just prior to the elution of arginine. These peaks, which we designate as pre-arginine 1 and 2, have not been described in

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Table 1
Tendon specific activity and aging

	Years of age						
	2(F)	13(F)	25(F)	38(M)	48(M)	61(M)	74(M)
^{3}H cpm X 10^2/mg	921.2 (100)	840.8 (91.3)	632.4 (68.7)	563.2 (61.1)	301.0 (32.7)	406.1 (44.1)	199.4 (21.7)
^{3}H cpm/μg hydroxyproline	948.0 (100)	884.5 (93.3)	635.9 (67.1)	556.2 (58.7)	313.3 (33.1)	404.2 (42.6)	204.2 (21.5)

Specific radioactivity of borohydride-reduced human flexor tendon as a function of age. Weighed samples were hydrolyzed as described in the text and aliquots of each hydrolysate were measured by liquid scintillation spectrometry and for hydroxyproline content. Results are expressed as count per minute (cpm) per milligram (dry-weight) of reduced insoluble collagen and per microgram of hydroxyproline in the hydrolysates. Figures in parentheses represent the per cent of specific radioactivity compared to 2 years of age. F: Female, M: Male.

other studies and were found to decrease significantly with age (table 2).

In similar fashion, the relative abundance of the other major components was determined and are listed in table 2. The detailed results show that: 1) The unknown component in fraction 9–17 (A) and the reduced aldehyde, dihydroxynorleucine (B) increased continuously with age; 2) N^{ϵ}-hexosyl-hydroxylysine (D), N^{ϵ}-hexosyllysine, aldolhistidine and dihydroxylysinonorleucine (E, F, G) increased until 38 years of age and subsequently, they decreased; 3) hydroxylysinonorleucine (H), histidinohydroxymerodesmosine (J), pre-Arginine 1 (K) and pre-Arginine 2 (L) were found to decrease with age.

These significant changes in concentration of the reducible compounds in human tendon cannot be ascribed to only one phenomenon and one may consider the following possibilities: 1) The increase in the content of the reduced crosslink precursor, dihydroxynorleucine, may reflect increased formation of this compound by lysyl oxidase or, alternatively, its decreased incorporation into the aldimine crosslinks; 2) the variation in concentration of the glycosylated compounds may reflect the population of carbohydra-

Table 2
Radioactive crosslinks and aging

	Years of age						
	2(F)	13(F)	25(F)	38(M)	48(M)	61(M)	74(M)
A	1.33	2.15	4.75	6.55	8.60	9.60	12.70
B	0.72	0.83	1.80	1.38	4.20	3.70	4.80
C	1.12	0.90	0.85	0.69	1.50	1.80	1.40
D	2.88	4.22	9.07	11.13	8.10	6.20	5.50
E, F, G	6.78	10.43	16.83	28.98	14.10	11.90	7.60
H	13.43	7.40	3.73	3.06	4.20	4.30	2.90
I	1.10	1.19	0.69	1.08	1.80	1.90	2.20
J	10.79	8.22	5.58	1.72	2.70	2.80	2.00
K	3.95	7.53	1.52	0.65	1.00	1.00	0.90
L	13.23	20.57	15.11	7.17	8.40	6.70	3.00

Age-dependent changes in the most prominent radioactive components eluted by ion-exchange chromatography (fig. 1). The data are expressed as the per cent of individual peak radioactivity compared to the total eluted radioactivity. Abbreviations used are those in table 1 and fig. 1.

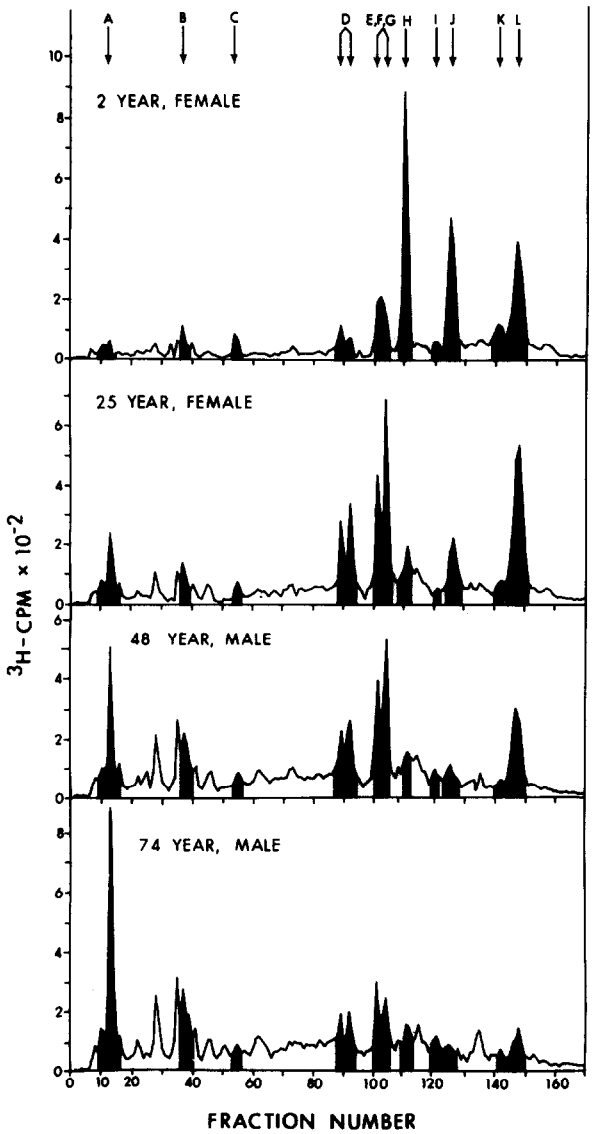

Fig. 1. Chromatographic patterns of the radioactive components in acid hydrolysates of reduced flexor tendon collagen of various ages. 5 X 10 cpm of each hydrolysate was applied to the amino acid analyzer. The conditions were: 0.9 X 69 cm column of Spinco UR-30 resin at 50°C; 0.25 M sodium citrate, pH 2.9; complex gradient to 0.4 M citric acid using 9-chambers: flow rate maintained at 80 ml per hr. Aliquots of 0.1 ml from the 2 ml of each effluent fraction were measured for radioactivity by liquid scintillation spectrometry. The peaks are: A, unknown; B, dihydroxynorleucine; C, hydroxynorleucine; D, N^{ϵ}-hexosylhydroxylysine; E, N^{ϵ}-hexosyllysine; F, aldolhistidine; G, dihydroxylysinonorleucine; H, hydroxylysinonorleucine; I, lysinonorleucine; J, histidinohydroxymerodesmosine; K, unknown (pre-Arginine 1); L, unknown (pre-Arginine 2).

tes and glycosaminoglycans in the tissue; 3) the substantial decrease in the aldimine crosslinks may reflect either their catabolism, their conversion to nonreducible compounds or their reduction in vivo. In this regard it is of interest that in mineralized collagens, isotope dilution studies showed that dihydroxylysinonorleucine is significantly reduced in vivo [8]. In contrast, others find that such reduction does not seem to occur in soft tissue collagen [12]. At any rate, whatever mechanisms of human connective tissue maturation are operative, the present studies strikingly demonstrate progressive, quantitative changes in both the incorporation of tritium into the reducible compounds and the dramatic change in their relative abundance as a function of chronologic age.

Acknowledgements

This work was supported by USPHS Grant AM-12683. We wish to thank the Department of Orthopaedic Surgery, The Jikei University School of Medicine, Tokyo, which provided us with the tendon samples.

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