PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 362-365 (1976)

Age-Related Alteration in Human Heart Collagen[1] (39397)

R. J. ZWOLINSKI, C. R. HAMLIN, AND R. R. KOHN

Institute of Pathology, Case Western Reserve University, Cleveland, Ohio 44106

Hamlin and Kohn (1) have reported a decreased rate of collagenase digestion of insoluble human tendon collagen with increasing age. Tendon was used as an abundant source of collagen that can be readily purified.

Aging in mammals is accompanied by a decline in efficiency of virtually all physiological processes (2-4). A hypothesis has been proposed (5) explaining the debilities of aging using the collagen cross-linking model. This hypothesis suggests that insoluble collagen found in parenchymatous tissue should exhibit age-specific alterations similar to that found in human tendon.

We now report on the presence of such age-specific alterations in insoluble human myocardial collagen.

Methods and materials. Heart tissue source. Hearts were obtained at autopsy from male accident victims. The hearts were selected from two age groups: (i) 10 hearts 18- to 28-yr-old (mature group); and (ii) 6 hearts 62- to 91-yr-old (old group). Hearts directly involved in disease processes were not used for study. Hypertrophied hearts and hearts showing gross evidence of infarcts and/or fibrosis were excluded. Heart samples were obtained 2-6 hr after death and were stored at −40° until processed. Studies with fresh tendon and with purified collagen showed that storage did not significantly affect digestibility. There were no differences in storage and handling of samples in the two age groups.

Heart collagen purification. All procedures were performed at 5° unless stated otherwise. The left ventricle, including the interventricular septum, was excised. The epicardium and endocardium were dissected away, along with any visible fat, valves, blood vessels, and connective tissue. Fifty grams (wet weight) of myocardium were then pressed under a 40,000-lb ram force in a hydraulic press (Model SP-215, Pasadena Hydraulics, Inc., El Monte, California). The pressed tissue was placed immediately in 500 ml of 0.15 *M* NaCl in 0.01 *M* Tris-HCl (pH 7.4) and shaken vigorously on an agitator until repeated washings with the NaCl–Tris solution became clear. This usually occurred after 5 days with NaCl–Tris changes every 12 hr. The residue was then similarly washed with 1.0 *M* NaCl in 0.01 *M* Tris–HCl (pH 7.4). A few drops of toluene were added to the NaCl–Tris stock solutions to prevent bacterial growth. At the time of each wash change, the suspension was passed through a No. 60 U.S. Standard Testing Sieve (The W.S. Tyler Co., Cleveland, Ohio) with an opening of 250 μm. The material remaining on the sieve was removed and macerated for 2 min with a mortar and pestle and was then resuspended in 500 ml of the appropriate NaCl-Tris solution. Finally, this material was washed twice in 500 ml of distilled water with sieving after each wash. The material remaining on the sieve was removed and frozen at −40° until further processing.

The fraction passing through the sieve was pooled for each heart sample processed. These pooled washes were centrifuged at 25,000-30,000 *g* for 120 min. The resulting supernatants were discarded and the precipitates were frozen at −40°, lyophilized, and stored at −40°. This fraction was subsequently analyzed for collagen so that an estimate of total myocardial collagen content as well as percentage collagen recovered could be obtained.

The material frozen after isolation on the sieve was lyophilized and treated as previously described (1, 6), with the following modifications: Lipid was extracted in three changes of petroleum ether for 1, 3, and 24 hr, respectively. A total of four washes with 1.0 *M* NaCl in 0.01 *M* Tris-HCl (pH 7.4) and six final washes with distilled water were performed. The material remaining

[1] This study was supported by United States Public Health Service Grants No. AG-00026 and No. HD00669.

after these procedures was frozen at −40° and lyophilized. This material constituted the purified human heart collagen (purified material).

Collagen content of purified material. Three aliquots of 20 mg of purified material, homogenized in 1 ml of distilled water (using a ground-glass homogenizer), were extracted twice with 5 ml of 5% TCA at 90° for 60 min. The extracts were centrifuged at 20,000 *g* for 15 min. The fraction isolated from the pooled washes passing through the sieve was treated similarly. Hydroxyproline content in aliquots of supernatant were determined by the method of Stegeman and Stalder (7). Collagen content was determined assuming 14% hydroxyproline by weight.

Collagen digestion. Clostridial collagenase was obtained from the Worthington Biochemical Corp. (Lot CLSPA 2KA, 440 U/mg) and prepared as previously described (6). The collagen digestions, with purified material containing 10 mg of collagen, were measured in a pH-stat as reported previously (1, 6), with the following modifications: The reaction volume was 15 ml and consisted of 10 mg of collagen, 400 units of collagenase, 2 ml of distilled water, and 13 ml of 0.1 *M* salt solution (0.05 *M* in $CaCl_2$ and 0.05 *M* in NaCl). The reaction proceeded for 45 min, at which time it was stopped by freezing in an acetone–Dry Ice bath at −70°. The reaction mixtures were then stored at −40° until further analysis. The determinations were thus of rate of digestion, or of amount digested per 45 min. Digestion curves are not linear over this time period (1), but the amount digested is a linear function of age (6). Control incubations were done on aliquots of three samples of purified collagen from both mature and old groups, excluding the collagenase. Also, incubations were done on three aliquots of collagenase, excluding the collagen. The controls were otherwise treated in the same manner as the experimental group.

Digestion product analysis. The reaction mixtures were thawed and centrifuged at 100,000 *g* for 60 min at 5°. The digestion products were passed through an ultrafiltration membrane (Amicon PM 10; Lexington, Mass.), using an ultrafiltration cell (Amicon Model 12) to exclude collagenase. Aliquots of the digestion product filtrates were hydrolyzed in 6 *N* HCl at 110° for 18 hr. The hydrolyzed samples were then analyzed for hydroxyproline content as above and for ninhydrin reactive material by the method of Rosen (8). L-Leucine was used as the standard for the ninhydrin determinations.

Collagen standards. Three aliquots of 23-yr-old (>95% collagen) and 88-yr-old (>95% collagen) purified human diaphragm-tendon collagen were hydrolyzed as above and analyzed for hydroxyproline content and ninhydrin-reactive material. Three aliquots of hot TCA-extracted 18-yr-old (31.1% collagen) and 91-yr-old (41.4% collagen) purified human heart collagen were treated similarly. A ratio of total micrograms per milliliter of hydroxyproline to total micrograms per milliliter of ninhydrin-reactive material was determined. This served as a standard against which to measure the digestion products for evidence that the digestion products were a result only of enzymatic hydrolysis of collagen.

Statistical analysis. The differences between the means were compared using the Student's *t* test.

Results. The collagen concentration of mature hearts is greater than old hearts on a wet weight basis (Table I). No age-specific differences exist in the percentage of collagen recovered and in the percentage of collagen present in purified material (Table I).

The digestion rates of mature collagen are much greater than that for old collagen (Table I). Additionally, the amounts of hydroxyproline and ninhydrin-reactive material, per milliliter of digestion product filtrate, are greater in mature than in old samples. Filtrates from the incubated collagen and collagenase controls contain ninhydrin-reactive material. Recalculating hydroxyproline to ninhydrin ratios, after subtracting control ninhydrin values from experimental values, gives ratios of 0.159 and 0.167 for filtrates from mature and old groups, respectively. Thus, on the basis of hydroxyproline content, the digestion products represent collagen at least as pure as that from tendon (Table II).

Digestion in collagen controls is minimal, does not involve hydroxyproline-containing protein, and does not show an age-difference (Table I). Hydroxyproline to ninhy-

TABLE I. Determinations in Mature and Old Myocardial Samples.

	Experimentals		Controls[a]		
	Mature	Old	Mature collagen	Old collagen	Collagenase
Sample number[b]:	10	6	3	3	3
Collagen content of myocardium (% wet wt)	0.63 ± 0.03[d]	0.44 ± 0.05	–	–	–
Total collagen recovered (% dry wt)	85.8 ± 1.2	82.0 ± 1.4	–	–	–
Collagen content of purified material (% dry wt)	39.1 ± 2.1	39.4 ± 2.6	–	–	–
Digestion (μl NaOH)[c]	737 ± 9[d]	549 ± 26	8.0 ± 1.3	9.5 ± 0.3	18 ± 1.6
Hydroxyproline content of filtrates (μg/ml)	70.3 ± 0.8[d]	54.5 ± 2.2	0	0	0
Ninhydrin-reactive material content of filtrates (μg/ml)	584.1 ± 14.9[d]	447.5 ± 17.2	74.5 ± 14.1	53.2 ± 6.2	67.3 ± 7.4
Hydroxyproline to ninhydrin ratios	0.121 ± 0.004	0.122 ± 0.002	0	0	0

[a] Collagen controls consist of 10 mg of collagen from purified material which was placed in the reaction vessel under identical conditions as the experimentals except for excluding collagenase. Collagenase controls consist of 400 units of collagenase in the reaction vessel under identical conditions as the experimentals except for excluding collagen.

[b] Three aliquots of each sample were determined and an average value of all aliquots was obtained. All values are mean ± SE.

[c] Denotes the amount of 0.01 *M* NaOH added to reaction mixture during 45-min incubation.

[d] Value significantly different ($P < 0.01$) from corresponding old experimental value.

drin-reactive material ratios are very similar in mature and old highly purified human tendon collagen, the digestion products from purified material of mature and old human myocardium, and collagen extracted by TCA from mature and old heart (Table II).

Discussion. Noncollagenous material is present in the digestion product filtrates, as evidenced by the ninhydrin values of the collagen and collagenase controls. However, comparing ratios of hydroxyproline to ninhydrin reactive material in the digestion product filtrates and in highly purified human tendon collagen indicates the contribution of noncollagenous material is trivial, and that virtually all of the digestion is the result of collagen degradation and not of nonspecific proteolytic activity in the two age groups.

The data demonstrate the presence of age-specific alterations in insoluble collagen of human myocardium. The digestion rates and changes with age are similar to those found in human tendon, as previously reported (1). This suggests that increasing stabilization of collagen with age may be generalized throughout the body. Although the nature of the molecular alterations in aging collagen is not known, it has been suggested that they consist of intermolecular cross-linking, and it is assumed that such alterations are manifested by the decreased digestion rates with collagenase.

TABLE II. Ratios of Hydroxyproline to Ninhydrin-Reactive Material from Different Collagen Sources.

Collagen source[a]	Ratio[b]
Mature human tendon	0.126 (0.122 to 0.129)
Old human tendon	0.126 (0.120 to 0.134)
TCA extract of mature purified material[c]	0.123 (0.120 to 0.125)
TCA extract of old purified material	0.129 (0.128 to 0.129)
Mature digestion product filtrate[d]	0.121 (0.105 to 0.128)
Old digestion product filtrate	0.122 (0.111 to 0.127)

[a] For each mature and old collagen source, three aliquots of one sample were determined.

[b] Values are means of aliquot values with the range given in parentheses.

[c] Purified material from human myocardium, containing 10 mg of collagen, was extracted with hot TCA. The TCA extract was analyzed for hydroxyproline and ninhydrin-reactive material.

[d] Digestion product filtrate from purified material of human myocardium.

Rat heart becomes less compliant with increasing age (9). It was suggested that connective tissue might be responsible for this change. Kohn and Rollerson (10) obtained data suggesting that the increased rigidity of human myocardium was due to increased rigidity of intercellular collagen. Osmotic swelling ability was used as a measure of this rigidity. It was postulated that this increased rigidity may be due to progressive formation of intermolecular bonds between collagen molecules.

Myocardial rigidity resulting from alterations in collagen could result in decreased myocardial contractility which may explain, in part, the physiologic decline in cardiac function known to occur with age (2).

Additional data are required to determine if sites of enzymatic cleavage are similar in the two age groups studied. Analysis of the unhydrolyzed digestion product filtrates would be useful in this regard.

Summary. A technique for the purification of insoluble human myocardial collagen is described. The digestion rate of old collagen with collagenase is much less than that of mature collagen. These findings are similar to those in human tendon collagen. It is suggested that progressive stabilization of collagen with age may be a generalized phenomenon, and responsible, in part, for the decline of myocardial function with age.

The authors gratefully acknowledge the assistance of Dr. Lester Adelson and Dr. Charles S. Hirsch of the Cuyahoga County Coroner's Office for providing the heart tissue.

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Received December 11, 1975. P.S.E.B.M. 1976, Vol. 152.