Analysis of Hydroxypyridine Cross-Links in Human Costal Cartilage Collagen

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Maturation of certain types of collagen is accompanied by the formation of trifunctional cross-links accomplished by hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP). HP and LP form intermolecular covalent bonds stabilizing collagen fibrils [7, 11, 12]. The content and ratio of hydroxypyridine links determine the structural and functional features and mechanical properties of the extracellular matrix of dense connective tissue [8]. Changes in the content and spectrum of cross-links in tissue collagens have been found in a number of hereditary collagen disorders [3-5, 7]. Costal cartilage is one of the target tissues in collagen disorders associated with thoracic deformations; it differs markedly in its structure and function from the better studied articular and intervertebral disc cartilages. Analysis of the hydroxypyridine cross-links responsible for structural specificities, as well as of the processing and fibrillogenesis of costal cartilage collagens is a pressing and as yet unsolved problem.

The aim of this study was to measure the HP and LP content in human costal cartilage specimens at various stages of normal ontogenesis and in connective-tissue diseases using high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Costal cartilage specimens (n=17) were obtained from patients with Ehlers-Danlos (EDS) and Marfan's (MS) syndromes complicated by pectus excavatum (PE) ("funnel breast"), with Ume's dystrophy complicated by pectus carinatum (PC), with isolated PE, and with isolated relapsing PE. Patients from the Chest Department of the Republican Pediatric Clinical Hospital and members of their families were examined at the Scientific Consultation Department of the Medical Genetics Research Center of the Russian Academy of Medical Sciences. The patients were divided into the following groups: 1) PE alone; 2) EDS combined with PE; 3) MS complicated by PE; 4) isolated relapsing PE; and 5) Ume's dystrophy with PC. Costal cartilage biopsy specimens were obtained during surgical correction of thoracic deformation. The samples were kept at -70°C. Control specimens of costal cartilage from subjects of various age groups without symptoms of connective-tissue disorders (n=12) were obtained not later than 4 h after clinical death was ascertained and stored at -70°C. Fetal costal cartilage specimens (n=2) were obtained during induced abortions performed for medical indications. Cartilage samples (less than 5 g wet weight) were separated from the perichondrium, minced with a scalpel, and subjected to hydrolysis in 6 N HCl in sealed glass vials at 110°C for 24 h. HP and LP were extracted from acid hydrolysates [10] by chromatography on columns packed with Filtrak cellulose (Germany). After elution with H_2O, HP and LP were freeze-dried and redissolved in loading buffer solution consisting of 100 mM ammonium acetate and 20 mM sodium octanesulfonate at pH 3.5. Chromatography was performed using the HPLC system,, lot 800, Jasco (Japan), which in-

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cluded two 880-PU pumps, an 821-FP fluorometer, an 802-SC control device, and an integrator from System Instrument Company (Japan). HP and LP were separated on the Altex Ultrasphere ODS column, 3 μ (7.5 cm×4.6 mm) preceded by passage through an Ultrasphere ODS precolumn (5 cm×4 mm, 5 μ). HP and LP were separated in a modified gradient system [2] at a flow rate of 0.5 ml/min. Solution A contained 20 mM ammonium chloride and 5 mM sodium octanesulfonate at pH 3.5. Solution B consisted of 75% acetonitrile and 25% of solution A with octanesulfonate final concentration 5 mM. HP and LP were separated using a linear gradient, starting from 90% solution A, whose concentration was reduced to 70% during 15 min. The solution A concentration was then reduced to 30% in 3 min and remained thus for 13 min, after which it was increased to the initial value within 3 min and remained unchanged for 8 min in order for equilibrium to be reached. The whole cycle took 41 min. HP and LP identification was based on their natural fluorescence at λ_{ex}=295 nm and λ_{em}=400 nm, taking into account the retention time in comparison with the prepared standards. Reference preparations of hydroxypyridine cross-links were isolated from type I collagen of bovine tendon (Sigma) and from costal cartilage and femoral bone samples obtained from a 30-year-old man who had died of an acute injury. HP was isolated from type I collagen after Fujimoto [9] using ion-exchange chromatography on a column with H^+ form phosphocellulose (Serva), 1.6×30 cm, this being followed by thrice repeated purification by gel filtration in a Toyopearl HW-40F column (Toyo Soda, Japan), 1.6×100 cm, at room temperature, using 10% acetic acid as an elutriator. Separation of HP from acid hydrolysate of human costal cartilage was performed after Eyre *et al.* [8]. The standard HP and LP mixture was obtained similarly from human femoral bone. The absorption spectra of the HP standard in 0.1 N HCl (pH 2.0) and 0.1 M potassium phosphate buffer (pH 7.4) were recorded with a Beckman DU-50 spectrophotometer (USA). The spectrum of the standard fluorescence at the mentioned pH values was checked on a Hitachi 850 spectrofluorimeter (Japan). The purity of the isolated HP standard was evaluated with a Biotronik LC-7000 amino acid analyzer (Germany) according to a routine program for acid hydrolysates. Analysis of the HP and LP mixture was performed in 0.3 M sodium citrate buffer solution (pH 4.49) at 55°C. Hydroxypyridine cross-links were detected by postcolumn derivation with ninhydrin. Collagens were mapped using electrophoresis of cyanogen bromide-cleaved fragments in polyacrylamide gel in the presence of sodium dodecylsulfate [13]. The ratio of type II to type I collagens was estimated after Amiel *et al.* [1]. Peptides α_2 (I) CB3.5 and α_2 (II) CB10.5 served as markers for type I and type II collagens, respectively. The following reagents were used in the study: deionized water purified in a Milli Q device (USA), hydrochloric acid (Fluka, Switzerland), sodium octanesulfonate (Sigma), cyanogen bromide and acetonitrile (Merck, Germany), acrylamide, sodium dodecylsulfate, methylene-bis-acrylamide, Coumassie R-250, ammonium persulfate, and amino acid standards (Serva). Other reagents were of Russian manufacture, chemically pure and/or superfine.

RESULTS

HP standards isolated from type I collagen of bovine tendon and human costal cartilage had a similar time of elution (53 min) from the column of amino acid analyzer, coming out between Phe and Hlys under standard conditions, and 14 min (between Tyr and Phe) when eluted with 0.3 M sodium citrate buffer at pH 4.49. Amino acid analysis of a standard mixture of bone-derived HP and LP eluted with 0.3 M sodium citrate buffer revealed two peaks differing in retention time by four minutes. The purity of the HP standard was confirmed by the absence of any other amino acids unrelated to HP in the course of amino

TABLE 1. Content of Hydroxypyridine Cross-Links in Costal Cartilage of Patients with Connective-Tissue Disorders

Patient	Disorder	Age, years	HP	LP
			nmol/mol collagen	
	Isolated PE			
T. K.		5	1.29	−*
P. D.		9	1.48	−
K. S.		9	1.43	−
M. M.		10	1.45	−
A. G.		11	1.09	
	MS + PE			
O. R.		13	1.04	−
K. T.		14	0.83	−
M. Yu.		14	1.48	2.25×10^{-2}
	EDS + PE			
G. Zh.		4.5	0.98	−
D. D.		5	1.66	1.82×10^{-2}
G. S.		7	2.35	9.34×10^{-2}
B. K.		10	1.09	−
M. T.		12	0.87	2.01×10^{-2}
V. A.		14	1.62	3.02×10^{-2}
	Relapsing isolated PE			
P. A.		9	0.92	1.87×10^{-2}
M. D.		15	1.67	1.69×10^{-2}
	Ume's dystrophy + PC			
T. Zh.		10	1.83	2.27×10^{-2}

Note. Asterisk: no LP, that is, $<0.1\times10^{-2}$ mol/mol collagen.

acid analysis in a standard system for analysis of protein hydrolysated.

HP standards isolated from type I collagen of bovine tendon and from human costal cartilage, as well as a standard mixture of HP and LP from human bone had similar maxima of the absorption spectra: λ=325 nm at pH 7.4 and λ=295 nm in 0.1 N HCl solution. The peaks of the fluorescence spectra were equal to 400 nm both at acid and at neutral pH, which is characteristic of 3-hydroxypyridines. These results enabled us to consider the obtained substances as HP and LP, respectively, and to use them as reference samples in HPLC.

During HPLC with a flow rate of 0.5 ml/min the retention periods for HP and LP were 13.84 and 14.74 min, respectively. The standard calibration curve for HP exhibited a linear form in the concentration range of 0.5 to 35 pM. The mean systematic error of analysis was 3.4%.

In costal cartilage as well as in articular cartilage only HP could be detected in the postnatal period. The content of HP in postnatal costal cartilage was equal to 1.34±0.33, according to our estimation. This result is in good agreement with the mean content of HP in articular cartilage of persons aged 24 to 80 [8]. In costal cartilage specimens examined by us the HP concentration tended to increase with age, reaching the maximal values by the age of 6. A similar tendency of ontogenesis-related changes of HP content was observed by Eyre *et al.* [6], who examined a large number of postnatal articular cartilage specimens and found the maximum HP concentrations in subjects aged 15 to 25. LP was detected in just solitary samples of fetal and postembryonal cartilage. The mean HP/LP ratio calculated from the ratio of areas under the respective chromatogram peaks in fetal costal cartilage samples was 23.47±5.33 (mean value ± standard deviation).

TABLE 2. Ratio of Type II to Type I Collagen in Costal Cartilage of Patients with Thoracic Deformations

Patient	Disorder	Age, years	Type II/I ratio
	Isolated PE		
P. D.		9	5.9
A. G.		11	6.0
	MS + PE		
K. T.		14	5.7
	EDS + PE		
G. Zh.		4.5	4.8
G. S.		7	5.0
M. T.		12	5.6
V. A.		14	5.0
	Relapsing isolated PE		
P. A.		9	4.2
M. D.		15	4.5
In health		6–41	5.65±0.87

Table 1 presents the results of a study of mature cross-links of collagen in costal cartilage specimens from patients with connective-tissue disorders. In isolated PE the HP content in costal cartilage remained within the normal range. However, LP was found in the costal cartilage of patients with isolated relapsing PE, in almost all the patients with EDS, in one with MS, and in one more with Ume's dystrophy. The LP content was particularly high in patient G. S. Two patients, K. T. and M. T., exhibited a reduced HP content in the cartilage. Table 2 presents the values of the type II to type I collagen ratio estimated by peptide mapping after cyanogen bromide cleavage. The ratio of type II to type I collagens in patients with EDS and MS complicated by PE, as well as in those with isolated PE, corresponded to the values in age-matched controls [13]. Nevertheless, type I collagen was found in cartilage regenerate formed after surgical correction in patients with recurrent PE, similarly as in embryonal costal cartilage.

Precise identification of HP and LP with the use of prepared standards and the agreement between the mean HP content in costal cartilage and the mean HP content in human articular cartilage estimated previously [8] permit us to disregard the modifications used in HPLC of collagen cross-linking as exerting no influence on the efficacy of HP and LP detection and quantitation.

The presence of LP in cartilage commonly not containing this type of cross-linking may be due to at least two factors: reduced lysylhydroxylase activity and the presence of type I collagen. The appearance of LP in costal cartilage of patients with isolated relapsing PE and the absence of this cross-linking in the cartilage of patients with isolated PE is due to the presence of type I collagen. The validity of this assumption is confirmed by the presence of peptide α_2 (I) CB3.5 on collagen peptide maps. The presence of type I collagen may be evidence of both extracellular matrix "embryonization" and of the presence of ossification sites in costal cartilage regenerate during relapsing PE. The ratio of type II to type I collagen in patients with PE concomitant with EDS and MS was within the normal range, and, hence, the presence of LP in the costal cartilage of such patients is most likely to be caused by lysylhydroxylase insufficiency. The marked LP increase in the cartilage of patient G. S. suggests a lysin hydroxylation deficiency, that is, type VI EDS. This is further confirmed by the data of clinical examinations. The reduced costal cartilage HP content observed in two patients is difficult to explain, although it undoubtly points to impaired processing of the cartilaginous collagens, which may be a second-

ary phenomenon related to an unknown biochemical defect. All these results, though referring to just solitary cases, should be taken into consideration because samples for investigation from human subjects are not easily available. There are virtually no data on the number of collagen hydroxypyridine cross-links in human costal cartilage in health and disease. Analysis of hydroxypyridine cross-links in costal cartilage samples may help understand the etiopathogenesis of the disorders observed in connective-tissue diseases complicated by thoracic deformations. According to published data, thoracic deformations are not a characteristic sign of the majority of EDS types, but analysis of a sample of patients hospitalized in the Chest Department has led us to the conclusion that EDS is often associated with complications and appears to contribute to PE/PC formation. The diverse patterns of changes in the content and spectrum of collagen cross-links in patients with EDS and MS most likely confirm the genetic heterogeneity of these nosological entities.

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EXPERIMENTAL BIOLOGY

Effect of T-activin on Macrophage 5-Nucleotidase Activity and Blood Cortisol Level as a Function of the Time of Day

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The long-standing investigation of immunomodulators of various chemical structure and origin has revealed that the reaction of the organism to an immunomodulatory influence involves complex relationships between phagocytosing cells and the neuroendocrine component of the regulation of immunity, in particular, between ecto-5-nucleotidase (5-n) activity in peritoneal exudate macrophages (PEM) and cortisol level [3,5].

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