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COMPOSITION OF PROTEOGLYCANS IN CARTILAGES OF GUNN RATS

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Summary

The ability of homozygous Gunn rats to conjugate trimethylacetic acid with glucuronic acid was significantly reduced from that of the heterozygous Gunn rats. This was interpreted as indicative of a reduced glucuronosyl transferase activity in the livers of the homozygous rats. However, the glucuronosyl transferases which promote the synthesis of chondroitin sulfate chains in cartilages are apparently unaffected. Proteoglycans extracted from costal, tracheal and xiphoid cartilages of homozygous rats were indistinguishable from the proteoglycans isolated from the same cartilages of heterozygous rats. Indeed, the chondroitin sulfate chains from the cartilages of the homozygous rats were of the same size as those from the cartilages of the heterozygous rats.

Comparison of the data obtained in this study with similar data on proteoglycans from bovine nasal septal cartilage revealed that the rat proteoglycan monomers were smaller than the bovine proteoglycan monomers. In addition, the protein cores of the rat proteoglycans had less histidine, arginine, proline, tyrosine, and phenylalanine and more serine and glycine residues than the protein core of the proteoglycan from bovine nasal septal cartilage.

Introduction

In 1938, Gunn [1] reported the discovery of a mutant strain of rats, which were jaundiced within 24 h after birth. They had an abnormally high level of free bilirubin in their circulation. The rats of this mutant strain are jaundiced

Abbreviations: A1—D1 fractions, the lowest 1/5 of the gradient.

because their livers are deficient in the glucuronosyl transferase which catalyzes the synthesis of bilirubin glucuronide [2] and glucuronides of other aglycones [3]. Data from breeding experiments show that the defect is a non-sex-linked recessive character [1,4].

It has also been reported that in the livers of the homozygous Gunn rats there are reduced levels of UDPglucosyl transferase activity [5] and of UDP-xylosyl transferase activity [6]. However, the latter deficiencies are not found in all colonies of Gunn rats [7]. As a possible explanation, Dutton [7] suggested that Gunn rats from different laboratories may express different genetic backgrounds. There is another possible explanation. Bilirubin is an inhibitor of a number of enzymes [8—14]. Its concentration in the circulation may vary with time. If, then, the activity of an enzyme in an homogenate of liver is examined when the concentration of free bilirubin is high, the activity of the enzyme may be low because of inhibition, and vice versa.

In a further description of the Gunn rat [4,15], it was reported that the homozygous animals did not grow at the same rate as the heterozygous animals.

The two observations, (1) deficiency of a glucuronosyl transferase in the livers of homozygous animals, which (2) did not grow at the same rate as heterozygous animals, raised a question. Is the slower rate of growth of homozygous rats related to the synthesis of abnormal chondroitin sulfate chains attached to the protein of the proteoglycan in their hyaline cartilages?

Proteoglycans of hyaline cartilages are complex macromolecules, the average molecular weight of those in bovine nasal septal cartilage is $2.5 \cdot 10^6$ [16]. They are composed of about 100 chondroitin sulfate chains and about 50 keratan sulfate chains, each of which is covalently attached to a protein of about 200 000 daltons [17]. Obviously, the biosynthesis of such complex macromolecules involves a coordinated interplay of many enzymes. Even in the synthesis of the chondroitin sulfate chains, which are composed of a repeating unit of D-glucuronic acid and *N*-acetyl-D-galactosamine-4- and/or -6-sulfate, two separate glucuronosyl transferases are utilized [18]. After the stepwise synthesis of the trisaccharide attachment region, consisting of galactosyl-galactosyl-xylosyl residues, on specific seryl residues of the protein core, the addition of the first glucuronosyl residue of the chondroitin sulfate chain is promoted by a glucuronosyl transferase which differs from the second glucuronosyl transferase which alternates with the transferase promoting the addition of *N*-acetylgalactosaminyl residues to extend the glycosaminoglycan chain.

Experimental procedures

Eighteen heterozygous and eighteen homozygous Gunn rats were used at about 60 days of age. The rats in each group were equally divided as to sex. They were fed a Teklab Mouse and Rat Diet ad libitum for one week before six representative rats from each group were checked as to their potential to conjugate trimethylacetic acid with glucuronic acid [19]. To this end, the rats were housed in individual metabolism cages for the quantitative collection of their urines during 24-h periods. While thus caged, they were deprived of food but had access to water. A week later, each of the rats received 25 mg of sodium

trimethylacetate in 1 ml of water by intraperitoneal injection. They were again segregated in the metabolism cages for 24 h for the collection of urine.

The concentrations of creatinine and of hexuronic acids in the urine samples were determined. Each of the 24-h specimens of urine was diluted to 50 ml with water. To 1 ml aliquots, 5 ml of a solution of picric acid * was added. After 30 min at room temperature, each of the solutions was diluted to 25 ml with water and the absorbance at 520 nm was determined. The latter was related to values of absorbance obtained when reference solutions of creatinine were similarly treated at the same time. The automated procedure of Heinegård [20] was used for the determination of hexuronic acids in the urines.

Subsequently, the rats were given an overdose of ether. At this time, the average body weight of the heterozygous rats was 149 g (range 118—180 g) and that of the homozygous rats was 135 g (range 106—185 g). The costal, tracheal, and xiphoid cartilages were removed. The cartilages were stripped of muscle and perichondrium before they were minced with a single-edged razor blade. The slices were about 1 mm thick. The pools of cartilage were extracted for 24 h at 4° C with 10 vols. of 4 M guanidinium chloride solution, which also was 0.05 M sodium acetate, 0.01 M Na · EDTA, 0.1 M 6-aminohexanoic acid, and 0.01 M benzamidine hydrochloride [21]. The pH of the extracting solutions was adjusted to pH 5.8 with acetic acid. The extracts were clarified by filtration through glass-wool and then by centrifugation at 4000 × *g* for 15 min at 4° C. The clarified extracts were dialyzed for 16—24 h at 4° C against 9 vols. of the extracting solution, which, however, did not contain the guanidinium chloride. The retentates, now at approx. 0.4 M guanidinium chloride, were put into associative gradients [16]. The A1 fractions thereof (the lowest 1/5 of the gradient) were in turn put into dissociative gradients [22]. The A1—D1 fractions (the lowest 1/5 of the gradient) were dialyzed against three changes of 100 vols. of 0.1 M potassium acetate and then against four changes of water at 4° C. The retentates were lyophilized.

The lyophilized A1—D1 preparations, also referred to as proteoglycan subunit preparations [1], were analyzed for content of protein [20], amino acids [23], hexuronic acids [20], and hexosamines [21]. Enough material was obtained only from the costal cartilages for analysis in the analytical ultracentrifuge. The sedimentation coefficients of the two components in the two samples were calculated as previously described [21].

The tissues after extraction with 4 M guanidinium chloride were washed with 10 vols. of water three times. They were then extracted at 4° C for 24 h with 10 vols. of 0.5 M NaOH. The extracts were exhaustively dialyzed against water at 4° C. The concentrations of uronic acids in the retentates were determined before lyophilization [20].

The chondroitin sulfate chains extracted from the cartilages with 0.5 M NaOH were compared as to size by gel permeation through columns of Sepharose 6B. The columns were prepared and used as suggested by Oegema et al. [21].

The guanidinium chloride and cesium chloride were purchased from

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* The picric acid solution was prepared just before use by mixing nine parts of a saturated solution of picric acid with one part of a 2.5 M solution of sodium hydroxide.

Schwartz/Mann; 6-aminohexanoic acid and benzamidine hydrochloride hydrate from Aldrich Chemical Co., Sepharose 6B from Sigma. All other chemicals were of analytical grade.

Results

Excretion of glucuronic acid in combination with trimethylacetic acid

It is clear from the data, summarized in Table I, that the homozygous rats excrete a significantly smaller amount of glucuronides/24 h period or per mg of creatinine than do the heterozygous rats. When challenged with trimethylacetic acid, the homozygous rats were able to conjugate and excrete, on the average, 22% of the dose of trimethylacetic acid in combination with glucuronic acid, whereas the heterozygous rats similarly handled 32% of the dose.

Characterization of the proteoglycan monomers from costal, tracheal, and xiphoid cartilages

Under the conditions employed for the extraction of the proteoglycans from the cartilages, 80—83% of the total uronic acid in the tissues (total is the sum of the amount extracted with 4 M guanidinium chloride plus that extracted with 0.5 M sodium hydroxide) was recovered in the 4 M guanidinium chloride extracts. In turn, 80%, or more, was recovered in the A1 fractions of the associative gradients. When these aggregate preparations were further fractionated by centrifugation in a dissociative gradient, 90% of the uronic acids was recovered in the A1—D1 fractions.

The compositions of the A1—D1 preparations from the cartilages of the homozygous rats were indistinguishable from those of like cartilages from the heterozygous rats. For example, Table II, the ratio μM hexuronic acids/mg of protein in the A1—D1 preparation from the tracheal cartilage of the homo-

TABLE I

CONJUGATION OF TRIMETHYLACETIC ACID WITH GLUCURONIC ACID

Six representative rats of each kind were used. Urine samples were collected for 24 h while the rats were resident in individual cages without food. At the beginning of the experimental period, each rat received 25 mg of sodium trimethylacetate in 1 ml of water by intraperitoneal injection. Mean values ± 1 S.D. are given. The differences are values for the additional amounts of glucuronic acid excreted in 24 h and per mg of creatinine in the experimental period.

Basal period			Experimental period			Differences	
Uronate (mg/24 h)	Creatinine (mg/24 h)	Uronate/ creatinine (mg/mg)	Uronate (mg/24 h)	Creatinine (mg/24 h)	Uronate/ creatinine (mg/mg)	Uronate (mg/24 h)	Uronate/ creatinine (mg/mg)
Heterozygous (Jj)							
6.8 ± 0.47	5.95 ± 0.35	1.14 ± 0.07	19.31 ± 1.36	6.03 ± 0.69	3.20 ± 0.61	12.50 (32.3%) *	2.07
Homozygous (jj)							
4.1 ± 0.06	4.70 ± 0.32	0.88 ± 0.06	12.84 ± 2.35	5.11 ± 0.45	2.51 ± 0.42	8.70 (22.4%) *	1.63

* Fraction of the dose of trimethylacetic acid conjugated was calculated, assuming that one mol of glucuronic acid was used/mol of trimethylacetic acid.

TABLE II

RATIOS OF HEXURONIC ACID TO PROTEIN AND OF GALACTOSAMINE TO GLUCOSAMINE IN PROTEOGLYCAN MONOMERS FROM COSTAL, TRACHEAL AND XIPHOID CARTILAGES

Cartilage	μM hexuronic acid/mg protein		Galactosamine/glucosamine	
	Jj	jj	Jj	jj
Costal	28.8	30.2	81.6	80.0
Tracheal	24.1	24.8	67.5	67.4
Xiphoid	17.6	19.9	56.4	56.0

zygous rats is 24.8 compared to 24.1 for the A1—D1 preparations from the tracheal cartilage of the heterozygous rats; the molar ratios of galactosamine to glucosamine are also the same, namely, 67.4 and 67.5, respectively.

On the basis of their efflux from an analytical column of Sepharose 6B, it is apparent that the chondroitin sulfate chains from the cartilages of the homozygous rats were of the same size as those from the cartilages of the heterozygous rats, Such a comparison of the chondroitin sulfate chains of the costal cartilages of the two kinds of rats is shown in Fig. 1.

The amino acid profiles of the protein in the A1—D1 preparations from the homozygous rats are also indistinguishable from those of the heterozygous rats (Table III).

Only from the costal cartilages were sufficient amounts of the A1—D1 preparations isolated so that these could be analyzed in the analytical ultracentrifuge. In each of the two samples two components were found. Their sedimentation coefficients were calculated to be 3.11 and 10.05.

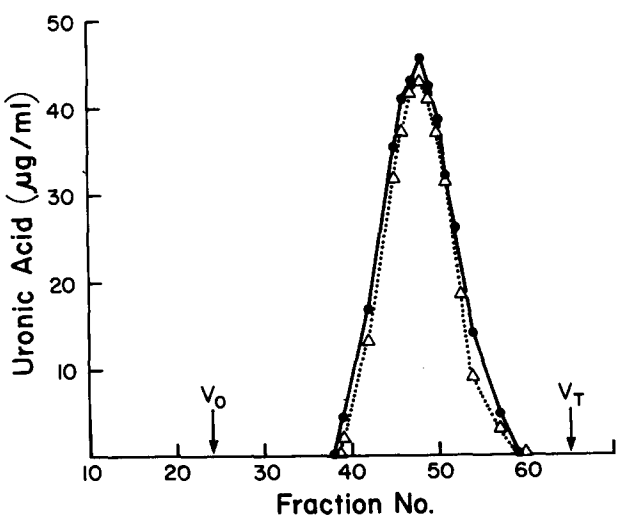

Fig. 1. Elution profiles of the chondroitin sulfate chains from an analytical column of Sepharose 6B when the source was the costal cartilage of heterozygous rats (●———●) and when the source was the costal cartilage of homozygous rats (△———△).

TABLE III

AMINO ACID COMPOSITIONS OF PROTEIN CORES IN PROTEOGLYCAN MONOMERS (A1—D1 PREPARATIONS)

Data are quoted as residues/1000 residues.

Amino acid	Costal cartilage		Tracheal cartilage		Xiphoid cartilage	
	Jj	jj	Jj	jj	Jj	jj
Lysine	21	21	14	13	25	25
Histidine	3	3	3	3	10	10
Arginine	18	18	13	15	14	15
Aspartic acid	69	67	70	70	75	74
Threonine	70	74	70	71	65	70
Serine	146	149	161	165	149	146
Glutamic acid	136	128	146	147	153	149
Proline	84	85	76	82	76	72
Glycine	177	176	184	183	169	167
Alanine	58	58	59	58	61	57
Half-cystine	6	6	6	6	5	6
Valine	62	63	62	58	64	70
Methionine	4	4	3	4	4	4
Isoleucine	29	30	28	26	27	26
Leucine	83	84	79	73	72	73
Tyrosine	9	8	6	6	9	9
Phenylalanine	26	27	20	18	21	24

Discussion

Although the homozygotes in our colony of Gunn rats are jaundiced at birth, or shortly thereafter, and are less able to utilize glucuronic acid for the production of glucuronides than are the heterozygotes, the data obtained in this study indicate that the proteoglycans in representative cartilage of the homozygotes at about 60 days of age are indistinguishable from the proteoglycans of the heterozygotes of the same age. Apparently, the glycosyl transferases in the cartilages of the homozygotes promote the initiation of the same number of chondroitin sulfate chains/unit weight of the protein core and the chondroitin sulfate chains are elongated to the same degree.

The rat at 60 days of age is still growing rapidly [24]. At this age one should expect the turnover of the macromolecules in the selected cartilages to be rapid. The fact that two components were found on ultracentrifugal analysis of the A1—D1 preparations from the costal cartilages may be a reflection of this. The slower sedimenting component, accounting for no more than 10% of the total, may be a breakdown product of the proteoglycan monomer in this tissue. The sedimentation coefficient, 3.11 S, of the more slowly sedimenting component is lower than that, 3.82 S, of the major product derived from the proteoglycan monomer of bovine nasal cartilage on incubation to the limit with cathepsin D of bovine liver and greater than that, 2.61 S, of the chondroitin sulfate peptide isolated from tryptic digests of the proteoglycan monomer of bovine nasal cartilage (Dziewiatkowski, D.D., unpublished observations).

It is noteworthy that the proteoglycan monomer with a sedimentation coefficient of 10 S is much smaller than proteoglycan monomers, 18—25 S, in

hyaline cartilages of other species, i.e. bovine nasal cartilage.

The amino acid compositions of the A1—D1 preparations from the cartilages of the Gunn rats, whether homozygous or heterozygous, are also divergent from those reported for A1—D1 preparations from hyaline cartilages of other species. For example, significantly fewer residues of histidine, arginine, proline, tyrosine and phenylalanine/1000 residues of amino acids are present in the protein cores of the A1—D1 preparations than in the protein core of similar preparations from bovine nasal cartilage [16]. On the other hand, preparations of the latter have fewer residues of serine and glycine/1000 residues of amino acids than the protein core of the A1—D1 preparations from the cartilages of the Gunn rats.

The divergence of the protein composition from that in comparable A1—D1 preparations from adult bovine nasal septum cartilage and the visualization of two components in the A1—D1 preparations from the costal cartilages of the young Gunn rats suggest the possibility that partial degradation had taken place before or during processing. Be that as it may, the A1—D1 preparations from the homozygous and heterozygous rats were concurrently subjected to identical manipulations. Since the preparations from the homozygotes are indistinguishable from those from the heterozygotes, one may conclude that the glycosyl transferases function as effectively in the cartilages of the homozygotes as they do in the heterozygotes of the Gunn strain of rats in our laboratory.

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