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Chondroitin sulfate glycosaminoglycans placenta

Changes in the Composition and Structure of Glycosaminoglycans in the Human Placenta during Development

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Extract

Glycosaminoglycans (acid mucopolysaccharides) are ubiquitous in their distribution in the body, yet information as to their biologic function is scanty. Studies of their structure and physical properties in solution suggest that they could function as gel filtration and exchange resins *in vivo*, thereby playing an important role in regulation of the passage of molecules through the ground substance of connective tissue. The glycosaminoglycan(s) (GAG) composition of the human placenta and the molecular structure of specific GAG has been studied by chemical, enzymatic, and physical methods at 12–18 weeks and at 40 weeks gestational age to explore this postulated structure-function relation.

The young placenta contained more GAG (222 mg/100 mg dry defatted tissue) than did the term placenta (155 mg/100 g dry defatted tissue). Sulfated GAG comprised 56% of the total GAG in the young placenta *versus* 74% in the term placenta due to increased concentrations of dermatan sulfate (25% term *versus* 13% young placenta) and heparan sulfate (22% term *versus* 15% young placenta). Chondroitin was a major component in the young placenta and comprised 22% of the total GAG, whereas the term placenta contained only 9%. Both young and term placenta showed similar quantities of hyaluronic acid and chondroitin 4- and 6-sulfates. Chondroitin sulfate from the young placenta differed from the polymer in the term placenta in that it contained a higher proportion of unsulfated dissaccharides. Differences were also found in the molecular structure of dermatan sulfate. Hyaluronidase digestion of purified dermatan sulfate from young placenta produced a 50% reduction in average molecular weight compared with a 30% reduction in the molecular weight of dermatan sulfate isolated from term placenta. The smaller molecular weight fragments of dermatan sulfate from young placenta indicate differences in molecular structure due either to the number of glucuronic acid substitutions or to their position in the polymer chain, or to changes in the concentration of hybrid molecules.

Speculation

The age-related changes in the composition and molecular structure of placental glycosaminoglycans will result in ground substance gels of differing physical properties. This could alter the transport of molecules through placental connective tissue and affect the rate of fetal growth.

Introduction

Ground substance is a histochemical descriptive term referring to the material of connective tissue which is neither cell nor fiber. It is the matrix in which cells and fibers are embedded and through which metabolites pass in exchanges between cells or between cells and the vascular system. Chemically, GAG (acid mucopolysaccharides) comprise a major and characteristic molecular species of this matrix [31]. Glycosaminoglycans may be described as protein-linked linear flexible polymers, composed of disaccharide repeating units containing 1 or more anionic groups per repeat unit. They occur in different tissues in typical patterns which may change with maturation and ageing. Information on their biologic function is scanty [26].

The structure of GAG as related to their physical properties has been extensively studied *in vitro*. Based on these types of experiments, several investigators have proposed that GAG play an important physiologic role in the regulation of transport exchange through the ground substance by virtue of their charge [32] and steric configuration [20].

All glycosaminoglycans are anionically charged at physiologic pH and act like polyelectrolytes; therefore, they may function as exchange resins *in vivo*, and bind compounds or ions which are cationically charged [25].

As polymers in solution, it has been shown that their steric configuration may exclude or sieve macromolecules traveling through the domain which they occupy. This property *in vivo* could influence the distribution of extracellular protein between various tissues, their colloid osmotic pressures, and the transport of macromolecules through and between tissue compartments [19].

There is no direct experimental evidence to establish this postulated structure-function relation *in vivo*. One way to explore this relation would be to establish a correlation between the composition and structure of GAG in a tissue as related to the transport function of that tissue. The placenta seemed a good organ to study experimentally for the following reasons: (*1*) it regulates the transport of metabolites between distinct anatomic and physiologic compartments [1], (*2*) its transport functions change with maturation and in certain pathologic states [21], and (*3*) if changes in chemical composition or the structure of component GAG are related to transport function, this may be reflected in the size of the infant or in his physiologic status at birth [21].

The purpose of this study is to establish base-line data on the glycosaminoglycan composition of the normal human placenta at 12–18 weeks gestational age as compared with the term placenta at 40 weeks, and to examine the molecular structure of specific GAG. Our data indicate that the concentrations of several GAG change dramatically as this organ matures, and that the molecular structure of dermatan sulfate and chondroitin sulfate is also altered.

Materials

Two pools of term placentas from 40-week pregnancies were obtained from the Perinatal Research Unit and the Department of Obstetrics of the Cleveland Metropolitan General Hospital. Initial analyses were carried out on three whole placentas designated as *pool A*. These data were further confirmed by analysis of 20% wet weight samples from 10 placentas designated as *pool B*. The clinical course in all 13 women was normal throughout gestation. Their infants were normal at birth and weighed between 2,700 and 3,850 g. The wet weight of the placentas ranged between 450 and 800 g.

Twenty-two placentas between 12 and 18 weeks of gestational age were obtained through legalized therapeutic abortions by abdominal hysterotomy at the University of Helsinki Women's Central Hospital. These specimens weighed between 4 and 22 g. The pregnancies had been interrupted for psychiatric reasons, none of the mothers had organic disease, and their fetuses appeared to be normal. The placentas were frozen, stored, and transported in Dry Ice. They were stored in our laboratory at −60° until analysis.

Methods

Whole young and *pool A* term placentas were processed in parallel for all succeeding steps. *Pool B* term placentas were analyzed separately using the same procedures except that only 20% of the weight of each placenta was used for the tissue pool.

The umbilical cord, fetal membranes, and the large vessels on the chorionic surface were removed by dissection. Tissues which remained were minced in a meat grinder and pooled. The total wet weight of the tissue pool from the young placenta was 152 g, from the term placenta *pool A*, 1,154 g, and term placenta *pool B*, 741 g.

The minced tissue pools were washed repeatedly with 75% ethanol and the wash removed by centrifugation at 2,000 rpm for 20 min. This process was repeated until the wash became colorless. The tissues were then defatted with acetone-ether (1/1, v/v) at 4° for 16–18 hr and the insoluble material was collected

by centrifugation at 2,000 rpm for 20 min at 4°. This process was repeated 4–5 times in young placenta and 10 times in the term placenta before cloudiness disappeared completely from the supernatant.

The tissue pools were then washed with 95% ethanol followed by diethyl ether and dried under a vacuum to a constant weight. The yields were 147 g of dry defatted tissue from the 3 term placentas, *pool A;* 102 g from the term placenta, *pool B;* and 17.2 g from the 22 young placentas. The dry defatted tissues were powdered in a Waring Blendor.

Isolation of Glycosaminoglycan

Next 60 g dry defatted term placenta *pool A* powder, 15 g term placenta *pool B* powder and 10.5 g young placenta powder were suspended in 0.5 M sodium acetate and digested with Pronase B [35] at 65°, and GAG were recovered from the digest by the procedure of Svejcar and Robertson [33] with only two minor modifications. Trichloroacetic acid was added to a concentration of 10% and GAG and glycopeptides were recovered from 80% ethanol. The precipitates were washed with 95% ethanol and diethyl ether and dried under a vacuum.

The dried precipitates were suspended in 0.01 M phosphate buffer in 0.02 M sodium chloride at pH 7 and digested with hog pancreas diastase [36], 6.5 mg/g tissue at 37° for 24 hr. Trichloroacetic acid, 100%, was added to the digest to a final concentration of 10%, the precipitate was removed by centrifugation at 2,000 rpm for 20 min at 4°, and GAG were recovered from the supernatant with 80% ethanol which contained 5% potassium acetate. This precipitate was washed successively with 95% ethanol and diethyl ether and dried under a vacuum.

To remove any remaining proteins, a second Pronase digestion was carried out and the GAG recovered as described above. The final precipitate was solubilized in 10% sodium acetate, then reprecipitated with 80% ethanol for 16 hr at 4°, and collected by centrifugation at 2,000 rpm for 20 min. The precipitate was washed sequentially with 95% ethanol and diethyl ether and dried under a vacuum to constant weight. The yields were as shown in Table I.

Table I. Yield for glycosaminoglycan (GAG) after second Pronase digestion

	Defatted tissue, dry wt, g	Crude GAG powder, dry wt, mg	Crude GAG powder, uronic acid content, mg
Term placenta			
Pool A	60	518	90
Pool B	15	240	24
Young placenta	10.5	128	23.3

Purification of Glycosaminoglycan

Column chromatography. Term placental *pool A* (195 mg), term placental *pool B* (228 mg), and young placental GAG mixtures (98 mg) (which contained 34 mg, 23 mg, and 18 mg uronic acid, respectively), were each dissolved in 5 ml distilled water and applied to columns (1.5 by 30 cm) of Dowex 1-X2, chloride form, 200–400 mesh resins. The columns were washed with 200 ml distilled water. Separation of the GAG mixture was achieved with a stepwise gradient of sodium chloride from 0.25 through 4.0 M. Twelve-milliliter aliquots of eluate were collected and the uronic acid content was measured. Only after elution was complete at a given chloride concentration was the concentration increased by 0.25 M increments and the elution repeated. Tubes which corresponded to each peak were pooled, dialyzed in viscose cellulose bags against distilled water to remove salt, and evaporated to dryness. This material was solubilized in 10% sodium acetate and the GAG recovered from 80% ethanol at 4° after 16 hr, washed with 95% ethanol and then diethyl ether, and dried under vacuum to a constant weight.

The recovery of uronic acid applied to the columns was 75% in the term placenta *pool A,* 92% in term placenta *pool B,* and 86% in the early placenta. After dialysis and precipitation, the recoveries were 53% term placenta *pool A,* 84% term placenta *pool B,* and 59% in the young placenta. The lower total recoveries in the term placenta *pool A* and the young placenta were not due to losses during dialysis but occurred because of the size and shape of the tubes that were used for precipitation and drying of these samples. We were unable to recover the dry powder completely from these vessels. In term placenta *pool B,* recoveries were higher because the samples were weighed and resolubilized in the same tube. As indicated by our analytic results, the GAG composition of the dry powder recovered from *pool A* was similar to the GAG composition of *pool B.* Therefore, we do not believe that differential losses of GAG occurred during isolation procedures.

Isolation of Pure Dermatan Sulfate

To carry out ethanol fractionations, additional crude GAG powder was prepared as described above from 6.3 g moisture-free defatted young placenta (yield 104 mg). This was combined with 22 mg material

which remained from the first preparation. The 123 mg so obtained was solubilized in 5% calcium acetate in 0.5 M acetic acid, as was 166 mg of GAG powder from the term placenta. The mixture of GAG was separated as calcium salts with concentrations of ethanol at 20%, 40%, and 60% as described by Meyer *et al.* [27]. Each fraction was treated by the alkaline copper precipitation method of Cifonelli *et al.* [6]. Dermatan sulfate was obtained as its copper complex from the 20% ethanol fraction and converted to its sodium salt. This precipitate weighed 19.7 mg from the term placenta and 8.3 mg from the young placenta.

The analytic data from the purified dermatan sulfate fractions are shown under *Results* in Table V.

Analytic Methods

Chemical methods. Uronic acid was determined by the borate-carbazole method of Bitter and Muir [2], and also by the naphthoresorcinol method of Pelzer and Staib [28] using glucuronic acid as the standard; hexose by the anthrone method using D-galactose as the standard; hexosamine by the method of Boas [3] omitting the Dowex 50W-X8 step; and the differentiation of glucosamine and galactosamine by the method of Ludowieg and Benmaman [24]. The samples for hexosamine determination were hydrolyzed in 2 N hydrochloric acid at 100° for 10 hr, and both D-glucosamine-HCl and D-galactosamine-HCl were used as standards. Sulfate was determined by the barium chloride turbidimetric method of Dodgson and Price [7], and 2-deoxy-2-sulfaminohexose was determined by the method of Lagunoff and Warren [18] using D-glucosamine-HCl as standard. Protein was measured by a modification of the method of Lowry *et al.* [23] in which the $CuSO_4$ was stabilized in alkaline solution with disodium EDTA [29].

Electrophoresis. Electrophoresis was performed on cellulose acetate membrane strips in 0.2 M zinc sulfate as described by Haruki and Kirk [12]. The strips were stained with 0.1% Alcian blue in 5% acetic acid-20% ethanol.

Enzymatic methods. Hyaluronidase digestions were carried out at 37° for 24 hr in NaCl-acetate buffer, pH 5.4, using 150 USP U enzyme/100 μg GAG as described by Saito [30]. Trichloroacetic acid, 100%, was added to the digest to a final concentration of 10%, and the precipitate was removed by centrifugation. Four volumes of absolute ethanol containing 1% potassium acetate were added to the supernatant, and the precipitate which formed at 4° after 16 hr was recovered by centrifugation at 2,000 rpm for 20 min. Both the precipitate, and the supernatant which was evaporated to dryness under reduced pressure, were solubilized in 0.001 M sodium hydroxide and analyzed for hexosamine and uronic acid. Standard GAG were used each time for control [38].

Chondroitinase ABC [39] digestions were carried out following *method I* of Saito [30]. The completeness of digestion was checked with GAG standards after 1- and 5-hr incubations at 37° using 0.2 U enzyme/100 μg GAG initially, with the addition of 0.1 U enzyme after 2.5 hr. Five hours were required to completely digest hyaluronic acid whereas the other GAG were completely digested after 1 hr. The total amounts of GAG in each fraction that were resistant or susceptible to digestion with chondroitinase ABC following 5-hr incubations were determined by recovering the undigested material as a precipitate from 80% ethanol which contained 1% potassium acetate after 16 hr at 4°. The supernatant was evaporated to dryness under a vacuum. Both the precipitate and residue from the supernatant were solubilized in 0.001 M sodium hydroxide and their uronic acid content was measured. The digests were then chromatographed on Whatman no. 1 (11.5 by 18.5 inch) paper from application spots 0.5 cm in diameter. Samples were first desalted by descending chromatography with 1-butanol-ethanol-water (52/32/16 v/v) for 24 hr, and the unsaturated disaccharides were then separated by descending chromatography in 1-butyric acid-0.5 M ammonia (5/3 v/v) for 48 hr at room temperature. The spots were identified under ultraviolet light by comparison with standard unsaturated disaccharides [40], cut from the paper, eluted in 2.0 ml of 0.01 M HCl at 50° for 10 min, and their ultraviolet absorption was measured in a DU[2] Beckman spectrophotometer [41] at 232 nm. The amounts of each unsaturated disaccharide recovered from the paper were calculated using the following millimolar absorption coefficients: 5.7 for nonsulfated disaccharides, 5.1 for 4-sulfated disaccharides, and 5.5 for 6-sulfated disaccharides.

Chondroitinase AC II digestions were carried on selected chondroitin sulfate fractions to differentiate chondroitin sulfate from dermatan sulfate using the methods outlined above [39]. The percentage of GAG digested with chondroitinase AC II was calculated from the ultraviolet absorption of disaccharides recovered after paper chromatography.

Measurement of molecular weight. The molecular weight of dermatan sulfate was measured by optical

mixing spectroscopy, a technique developed for analysis on quasielastic light scattering, in which the spectroscopic distribution of Rayleigh-scattered light is measured with allowance for calculation of diffusion coefficients and hence molecular weights. Two-milligram quantities of purified dermatan sulfate were dissolved in 20 ml distilled deionized water. The heterodyne Rayleigh spectrum of these solutions was recorded in a Brice Phoenix light-scattering cell no. C-105 [42] at room temperature (27°) using a laser heterodyne spectrometer which has been described in detail [14]. Weight average molecular weights were obtained by interpolation from known standards. A preliminary report of the procedure for the measurement of molecular weights of highly sulfated GAG has appeared [13]. A more detailed molecular weight study using line shape computer analysis is nearing completion and will be published.

Results

Column Chromatography

Chemical and enzymatic analysis of GAG fractions obtained by column chromatography from the young and term placenta are presented in Tables II and III.

The yield of GAG, as percentage of distribution of uronic acid recovered from the columns, differed in the young and term placenta in the 0.5 M and 1.5 M fractions (Table II). The 0.5 M fraction was larger in the young placenta as compared with the term placenta, whereas the reverse was true in the 1.5 M fractions. The mixture of GAG applied to the column was

Table II. Analytic data for young and term placental glycosaminoglycan fractions from Dowex 1 column[1]

Fractions M NaCl Molarity	Yield[2] (%)		Uronic Acid		Hexose (moles/mole of total hexosamine)		Sulfate		Sulfamine-hexose		Glucosamine to Galactosamine ratio		Borate to Naphthoresorcinol ratio		After Hyaluronidase Digestion Remaining[3] (%)		Borate to Naphthoresorcinol ratio	
Young Placenta																		
0.25	4.91		0.15		1.39		0.		0.		57:43		1.74		100			
0.5	26.99		0.99		0.46		0.		0.		64:36		6.04		16		0.51	
0.75	10.25		1.39		0.40		0.53		0.20		67:33		5.78		61		3.83	
1.0	9.49		1.29		0.33		0.77		0.18		86:14		8.05		66		3.16	
1.25	10.10		1.50		0.24		1.28		0.05		25:75		6.20		35		2.65	
1.5	28.11		1.12		0.32		1.47		0.		7:93		2.21		42		0.78	
1.75	7.01		1.16		0.37		1.37		0.		10:90		1.49		51		0.59	
2.0	1.99		1.28		0.27		1.67		0.		13:87		1.29		66		0.54	
3.0	0.32		1.36		1.22		2.80		0.		35:65		1.29		-		-	
4.0	0.78		0.71		0.65		1.19		0.		94:6		3.03		-		-	
Term Placenta	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
0.25	3.93	3.42	0.16	0.12	1.54	1.00	0.06	0.03	0.	0.	79:21	86:14	0.98	3.86	100	100	-	-
0.5	13.61	12.53	0.97	0.80	0.69	0.73	0.15	0.01	0.	0.	74:26	57:43	3.70	2.87	31	22	0.83	0.25
0.75	11.16	12.58	1.41	1.65	0.79	0.54	0.64	0.63	0.10	0.16	77:23	64:36	4.34	4.86	75	60	4.36	4.04
1.0	13.87	14.04	1.39	1.59	0.62	0.32	1.10	0.83	0.34	0.39	84:16	83:17	5.61	7.09	79	76	4.04	5.22
1.25	10.74	8.48	1.73	1.57	0.50	0.33	1.23	1.32	0.11	0.13	49:51	13:87	6.64	4.82	51	40	2.43	3.09
1.5	37.09	35.89	1.15	1.27	0.34	0.20	1.44	1.36	0.	0.	14:86	0:100	1.98	1.95	57	48	0.88	0.95
1.75	6.64	9.29	1.04	1.26	0.40	0.20	1.44	1.48	0.	0.	10:90	0:100	1.11	1.37	76	69	0.83	0.84
2.0	1.80	1.86	0.81	0.93	0.61	0.29	1.37	1.37	0.	0.	26:74	12:88	0.89	1.01	85	80	0.64	0.64
3.0	0.38	1.72	0.68	0.98	1.18	0.53	1.72	1.31	0.	0.	59:41	20:80	1.01	1.35	-	79	-	0.63
4.0	0.90	0.18	0.90	-	0.72	-	0.51	-	0.	-	59:41	-	1.77	-	-	-	-	-

[1] A: sample pool of 3 whole placentas; B: sample of 10 placentas using 0.20 wet weight of each for pool.

[2] Percentage of distribution for total glycosaminoglycan recovered from columns after dialysis and precipitation with 80% ethanol based on borate carbazole uronic acid content.

[3] Percentage remaining: $\frac{\text{borate uronic acid content after digestion}}{\text{total borate uronic acid content}} \times 100.$

Table III. Chondroitinase ABC and AC-II digestion of young and term placental glycosaminoglycan fractions from Dowex 1 column[1]

Fractions NaCl Molarity	Yield % (2)		% of total GAG Digested(3) Chondroitinase AC-II		Chondroitinase ABC	Distribution of Digested GAG (% of total uronic acid in each fraction)(4) unsaturated disaccharides non-sulfated		4-sulfated		6-sulfated		Hyaluronic Acid	
Young Placenta													
0.5	26.99		-		81	51		0		0		30	
0.75	10.25		-		42	33		2		trace		7	
1.0	9.49		-		39	18		trace		10		11	
1.25	10.10		70		70	15(16)[5]		9(8)		46(46)		0(0)	
1.5	28.11		69		100	5(5)		37(10)		58(54)		0(0)	
1.75	7.01		53		100	trace (8)		55(5)		45(40)		0(0)	
Term Placenta	A	B	B	A	B	A	B	A	B	A	B	A	B
0.5	13.61	12.53	-	67	75	34	41	0	0	0	0	33	34
0.75	11.16	12.58	-	28	39	19	19	0	0	0	0	9	20
1.0	13.87	14.04	-	20	24	7	6	trace	trace	1	3	12	15
1.25	10.74	8.48	62	61	61	11	8(8)	13	18(17)	26	35(37)	11	0(0)
1.5	37.09	35.89	58	100	100	0	1(2)	54	54(15)	46	45(41)	0	0(0)
1.75	6.64	9.29	25	100	100	0	0(0)	72	73(7)	28	27(18)	0	0(0)
2.0	1.80	1.86	-	78	100	0	0	58	75	20	25	0	0
3.0	0.83	1.72	-	-	76	-	0	-	52	-	24	-	0

[1] A: sample pool of 3 whole placentas; B: sample pool of 10 placentas using 0.20 wet weight of each for pool.

[2] Percentage distribution of total glycosaminoglycan recovered from Dowex 1 columns after dialysis and precipitation with 80% ethanol based on borate carbazole uronic acid content.

[3] Each fraction was digested with chondroitin ABC for 5 hr, and the undigested material was precipitated with 80% ethanol at 4° for 16 hr. Borate uronic acid was measured in the precipitate and supernatant. Chondroitin AC II digestions were carried out for 5 hr and the percentage of digested glycosaminoglycan calculated from the ultraviolet absorption of disaccharides recovered after their separation by chromatography.

[4] The concentration of unsaturated disaccharides was calculated from their ultraviolet absorption after their separation by paper chromatography. Hyaluronic acid was calculated as the difference of the borate uronic acid content of the total chondroitinase ABC-digested glycosaminoglycan and the recovered unsaturated disaccharide.

[5] Results of chondroitinase AC II digestion are shown within parentheses.

resolved on the basis of anionic charge which is best correlated with the total sulfate content (Table II). The term placenta had a higher proportion of sulfated GAG than did the young placenta, comprising 71% of the total GAG in the term *versus* 58% in the young, as judged by GAG eluted at 1.0 M NaCl or above.

Based on the ratio of glucosamine to galactosamine, total sulfate, sulfaminohexose, and hexose content (Table II), each fraction from the column contained a mixture of two or more GAG. This was confirmed by cellulose acetate electrophoresis. Fractions 0.25–0.5 M showed glycopeptide, hyaluronic acid, and undersulfated chondroitin. Fractions 0.75–1.25 M contained heparan sulfate and chondroitin sulfate isomers while fractions 1.5 through 2.0 M contained dermatan sulfate and chondroitin sulfate isomers. The 3.0 and 4.0 M fractions contained increasing amounts of hexose, glucosamine, and sulfate, which suggested the presence of a keratan sulfate-like material.

Characterization and Quantitation of Component Glycosaminoglycan in Dowex 1 Fractions

In order to quantitate the concentration of specific GAG in each fraction, enzyme digestions were carried out using bovine testicular hyaluronidase and chondroitinase AC II and ABC. Chemical analyses and electrophoresis were done on the GAG which were enzyme resistant or digestible, and the proportion (percentage) of a specific GAG in each fraction was calculated based on these data.

Fraction 0.25 M. One hundred percent of the material was hyaluronidase resistant. The precipitate contained a high hexose content, little uronic acid, no sulfate, and was designated as glycopeptide because 13% of its dry weight was composed of protein.

Fraction 0.5 M. The very low sulfate content in this fraction indicated that it contained mainly nonsulfated GAG. Hyaluronidase and chondroitinase ABC

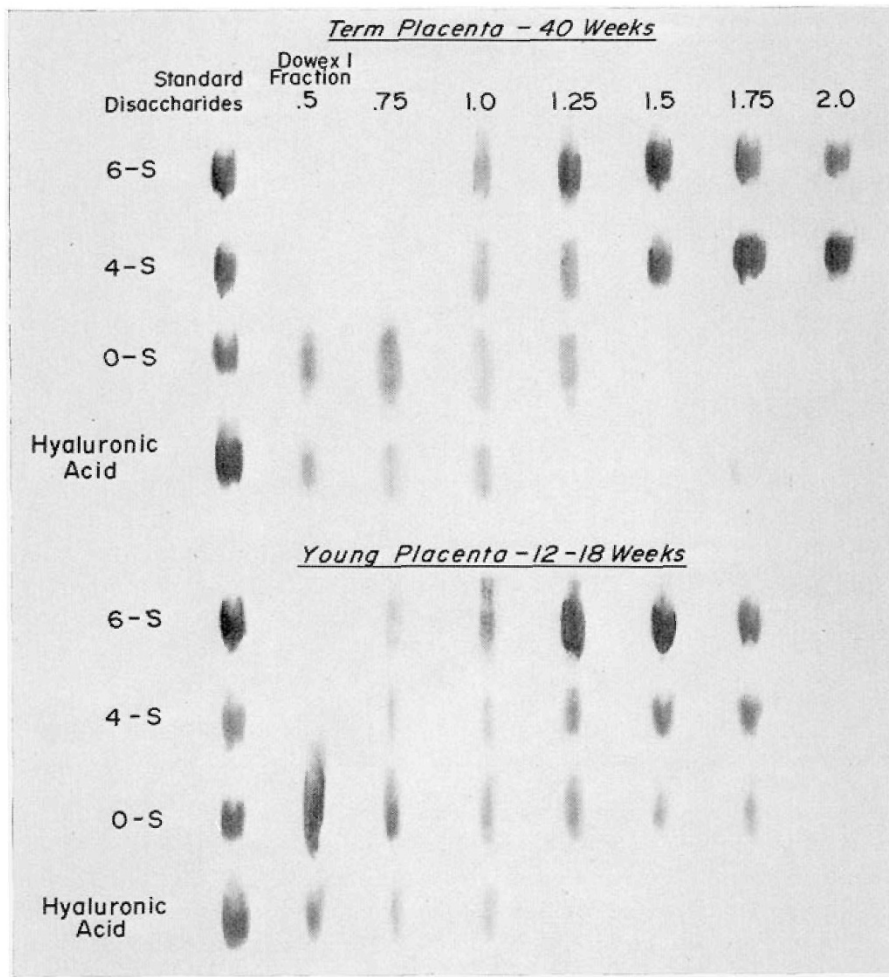

Fig. 1. Chromatography of unsaturated disaccharides after chondroitinase ABC digestion (tracings of the original chromatograms).

digestions yielded similar amounts of undigested material (Tables II and III). The enzyme-resistant fraction was considered to be glycopeptide despite the low carbazole-naphthoresorcinol ratio because digestion with chondroitinase ABC eliminated dermatan sulfate from the precipitate. In addition, only a single band corresponding to standard hyaluronic acid was found on cellulose acetate electrophoresis. Paper chromatography of the chondroitinase ABC digest demonstrated two spots (Fig. 1), which corresponded to the standard nonsulfated disaccharide, and to that obtained with standard hyaluronic acid degraded with the same enzyme. Nonsulfated chondroitin was calculated on the basis of the nonsulfated disaccharide recovered by the paper chromatography and comprised 51% of the total uronic acid of this fraction in the young placenta *versus* 34% and 41% in the term placenta (Table III). The difference between total chondroitinase ABC-digestible materials and nonsulfated chondroitin was considered to be hyaluronic acid (Table III) and was 33% and 34% in the term placenta *versus* 30% in the young placenta.

Fractions 0.75–1.0 M. The high glucosamine and sulfaminohexose content of these fractions indicated that they contained heparan sulfate. The total sulfate to hexosamine ratio was low, which suggested either an undersulfated heparan sulfate or the presence of other under- or nonsulfated GAG (Table II). After digestion with hyaluronidase, the borate to naphthoresorcinol ratio remained high, which eliminated dermatan sulfate as the resistant GAG in the precipitate (Table II). This was confirmed by chondroitinase ABC digestion which proved clearly that the enzyme-resistant GAG was heparan sulfate which comprised 76 and 69% of the total uronic acid in the term placenta and 59% in the young placenta (Table III).

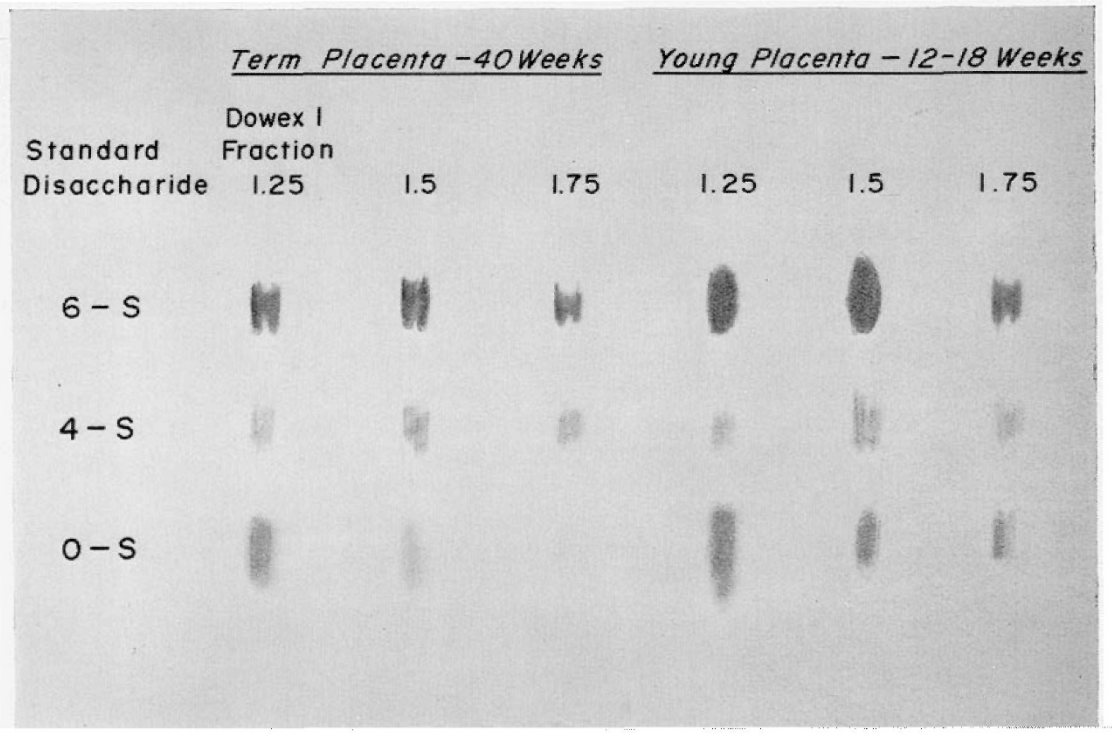

Fig. 2. Chromatography of unsaturated disaccharides after chondroitinase AC II digestion (tracings of the original chromatograms).

The major disaccharide isolated and recovered after chromatography of the chondroitinase ABC digest was a nonsulfated disaccharide in both the term and young placenta (Table III), with smaller quantities of 6-sulfated disaccharide, and only trace amounts of 4-sulfated material (Fig. 1).

Fraction 1.25 M. The increase of galactosamine indicated that the major GAG in this fraction were the isomers of chondroitin sulfate. The presence of sulfaminohexose indicates the presence of heparan sulfate (Table II). There were no differences found in the susceptibility of the GAG in this fraction to digestion with hyaluronidase and chondroitinase AC II and ABC, which indicates that the enzyme-resistant GAG was heparan sulfate (Tables II and III).

Three unsaturated disaccharides were obtained by the paper chromatography after chondroitinase AC II and ABC digestion (Figs. 1 and 2). The susceptibility to digestion with chondroitinase AC II shows clearly that all of the 4-sulfated disaccharides were derived from chondroitin 4-sulfate (Table III).

The principal GAG in this fraction was still heparan sulfate in the term placenta with smaller amounts of chondroitin 6-sulfate, nonsulfated chondroitin, and hyaluronic acid. By contrast, the major component in the young placenta was chondroitin 6-sulfate; heparan sulfate and nonsulfated chondroitin contributed smaller amounts (Table III).

Fractions 1.5–1.75 M. Galactosamine was the principal hexosamine in these fractions (Table II) and their GAG were completely digested to unsaturated disaccharides by chondroitinase ABC, which indicated that they were isomers of chondroitin sulfate (Table III). Of the GAG, 42–76% were resistant to hyaluronidase and chondroitinase AC II (Table II and III). These isomers showed a low borate to naphthoresorcinol ratio with no sulfaminohexose detected, which indicates that the hyaluronidase-resistant isomers were dermatan sulfate (Table III). The major disaccharides recovered after digestion with chondroitinase ABC were 4- and 6-sulfated compounds, whereas after chondroitinase AC II digestions, 6-sulfated disaccharides predominated. Hyaluronidase- and chondroitinase AC II-resistant GAG were designated as dermatan sulfate. The 4-sulfated and 6-sulfated disaccharides recovered after chondroitinase AC II digestion were derived from chondroitin 4-sulfate and 6-sulfate. Small quantities of nonsulfated disaccharides were present in the 1.75 M fraction in the young placenta whereas the term placenta contained none (Fig. 2). A light spot of very slow R_F was present in each fraction in both the term and young placenta. These were considered to be di-

sulfated disaccharides but their concentration was so small that they were not considered in this compositional analysis.

In the term placenta, dermatan sulfate composed 57% and 49% of these fractions and chondroitin 6-sulfate 43% and 36%, whereas in the young placenta chondroitin 6-sulfate comprised 52% of the total with smaller amounts of dermatan sulfate (35%). Only small amounts of chondroitin 4-sulfate were found in either term and young placenta.

Fraction 2.0 M. In both the term and young placenta this fraction showed material resistant to digestion with hyaluronidase with a low borate-naphthoresorcinol ratio which indicates the presence of dermatan sulfate (Table II). Sufficient material for chondroitinase digestion was not available in the young placenta. In the term placenta, 78% of the material was digestible with chondroitinase ABC and the major disaccharide was 4-sulfated. Approximately 66% of this fraction was attributed to dermatan sulfate in the young placenta on the basis of resistance to hyaluronidase and borate-naphthoresorcinol ratio (Table II). In the term placenta dermatan sulfate comprised 58 and 75% on the basis of chondroitinase ABC digestion. (Table III, Fig. 1) In addition, in the term placenta, the concentration of glucosamine and hexose increased, with decreased content of uronic acid suggesting that a keratan sulfate-like material was eluted with this fraction. No further characterization of this compound was carried out because of the limited amounts available for analysis.

Fractions 3.0–4.0 M. As the molarity increased, the eluates contained increasing quantities of glucosamine and hexose with smaller molar ratio for uronic acid, which suggested the presence of keratan sulfate-like compounds. Insufficient sample was available for further characterization of these fractions.

Table IV summarizes the composition of GAG in the term and early placenta. The amounts of each GAG identified in the column fractions have been summed to give this composite picture.

The GAG composition is presented as percentage of distribution of total GAG recovered from the columns after dialysis and reprecipitation of the GAG. Definite differences were found. The early placenta had a higher proportion of nonsulfated chondroitin, whereas the proportions of dermatan and heparan sulfate were increased in the term placenta. Chondroitin 6-sulfated was the principal hyaluronidase-susceptible chondroitin sulfate isomer in both the young and term placenta, with only small quantities of chondroitin 4-sulfate detected. Overall, the young placenta contained more GAG than did the term placenta, but the term placenta had appreciably higher proportions of hyaluronidase-resistant sulfated GAG. There appeared to be a reciprocal relation between the proportion of nonsulfated chondroitin and the concentrations of dermatan and heparan sulfate.

Structural analyses of dermatan sulfate from young and term placenta. The chemical composition and enzymatic analysis of dermatan sulfate isolated from the term and young placenta is given in Table V, and compared with standard dermatan sulfate isolated from hog mucosa. These data indicate that the GAG isolated from the placenta was highly purified dermatan sulfate. After digestion with testicular hyaluronidase no differences were detected by chemical and enzymatic analysis. Molecular size as measured by optical mixing spectroscopy showed a striking reduction in the molecular weight of the dermatan sulfate isolated from

Table IV. Glycosaminoglycan composition in normal term and young placenta

	Uronic acid in dry defatted tissue, mg/100 g	Recovery of uronic acid from Dowex 1 column		Distribution, %[1]							
		In eluate, %	After dialysis and precipitation, %	Glycopeptide	Hyaluronic acid	Nonsulfated chondroitin	Chondroitin 6-sulfate	Chondroitin 4-sulfate	Dermatan sulfate	Heparan sulfate	Keratan sulfate
Term placenta[2]											
Pool A	150	75%	53%	8.42	8.33	8.90	22.21	1.40	26.37	23.33	Some
Pool B	160	92%	84%	6.55	8.89	9.77	20.83	7.47	24.33	21.56	Some
Young placenta	222	86%	59%	10.04	9.86	22.45	24.26	4.18	13.58	14.77	Some

[1] Each Dowex 1 chromatographic fraction was studied chemically before and after digestion with hyaluronidase, as well as chondroitinase ABC and AC II. The identity and amount of specific GAG were calculated from these data.

[2] *pool A:* sample pool of 3 whole placentas; *pool B:* sample of 10 placentas using 0.20 wet weight of each for pool.

Table V. Analyses of placental dermatan sulfate (DS) before (A) and after (B) treatment with testicular hyaluronidase[1]

	Yield, %[2]	Hyaluronidase resistant, %	Borate to naphthoresorcinol ratio	Weight, %			Unsaturated disaccharide after chondroitinase ABC digestion			Molecular weight average ± SE
				Hexosamine	Uronic acid	Sulfate	Disulfated	6-sulfated	4-sulfated	
Hog mucosal DS										
A			0.77	28	26 (0.87)	23 (1.51)	7.8	4.7	87.5	27,900 ± 1,800
B		97	0.69	33	32 (0.87)	24 (1.33)	8.8	2.4	88.8	24,500 ± 1,800
Term placental DS										
A	14.82		0.76	33	31 (0.89)	24 (1.37)	0.9	6.4	92.7	23,400 ± 1,500
B		94	0.75	26	27 (0.98)	19 (1.37)	0.5	0.9	98.6	15,700 ± 1,500
Young placental DS										
A	8.75		0.73	22	27 (1.12)	18 (1.48)	1.5	6.7	91.8	21,700 ± 1,500
B		97	0.70	27	27 (0.91)	19 (1.34)	0.5	0.5	99.0	10,000 ± 1,500

Figures in parentheses are data expressed on molar ratio basis to hexosamine.
Percentage of total uronic acid in the crude placental GAG based on borate carbazole uronic acid.

the placenta, whereas hog mucosal dermatan sulfate was resistant to the action of this enzyme. The average size of the polymer was decreased by 50% in the young placenta and by 30% in the term placenta.

Discussion

These data show that age-related changes occur in the composition and structure of GAG in the human placenta.

The young placenta (12–18 weeks) and term placenta (40 weeks) both contain hyaluronic acid, chondroitin 6-sulfate, chondroitin 4-sulfate, nonsulfated chondroitin, dermatan sulfate, and heparan sulfate, but in differing proportions. Sulfated GAG comprised 56% of total GAG in the young placenta as compared with 74% in the term placenta with differences accounted for by an increase in hyaluronidase-resistant GAG and dermatan and heparan sulfate. Only small amounts of chondroitin 4-sulfate were detected. In addition, analytic data suggest the presence of small quantities of keratan sulfate-like GAG.

Calatroni and Di Ferrante [5] reported similar results for the GAG composition of term placenta except for larger amounts of chondroitin 4-sulfate. This discrepancy may be due to the methods used to identify chondroitin 4-sulfate. They used infrared spectral analysis which does not differentiate mixtures which contain 4-sulfated isomers of chondroitin from dermatan sulfate while the enzymatic methods used in this study clearly separate mixtures of these two isomers.

The only published study which compares the GAG composition of young and term placenta was by Lovell *et al.* [22] who did not detect heparan sulfate in either the term or young placenta, reported no age-related change in the concentrations of dermatan sulfate, but did show that the young placenta contained more undersulfated chondroitin 6-sulfate. These studies were carried out on the central area of the placenta midway between the decidua and chorionic plate while we used the whole tissue. The difference in results may be attributable to differences in methodology but not to tissue sampling since Takeuchi and Schafer [34] showed that the midportion of the placenta contained heparan sulfate.

Our analysis identified two types of chondroitin polymers in the placenta. Over 76% of the nonsulfated disaccharides were recovered from the 0.5 M and 0.75 M column fractions which contained little or no sulfate. The young placenta contained more of these unsulfated chondroitin chains than did the term placenta. Chondroitin 6- and 4-sulfate isolated from the higher salt column fractions were found to be hybrid molecules composed of sulfated and nonsulfated disaccharides. The young placenta showed a larger proportion of chondroitin sulfate polymers with this structure than did the term placenta.

Kaplan and Meyer [16] reported that human aorta contained hyaluronic acid, heparan sulfate, dermatan sulfate, and chondroitin 6-sulfate but no chondroitin 4-sulfate. This striking similarity in the overall GAG composition of both tissues is also paralleled by time dependent changes in their composition. Both tissues show decreasing total GAG content as they age with proportional increases in sulfated GAG. The increase in sulfated GAG in the placenta is due to increased concentrations of dermatan sulfate and heparan sulfate, with reciprocal decreases in the concentrations of

nonsulfated chondroitin. The aorta shows similar age-related increases of heparan and dermatan sulfate concentrations with decreased concentrations of chondroitin 6-sulfate and hyaluronic acid. Because the placenta is a highly vascular organ, the similarity of GAG composition to the aorta may simply reflect this fact. Although the patterns of specific GAG are different, striking age-dependent changes have also been reported for human costal cartilage [15] and skin [4], which suggests that GAG metabolism is related to biologic aging in many tissues.

Age-dependent changes in the structure of a specific GAG have not been previously described. Dermatan sulfate, the GAG selected for structural analysis in this study, is composed of $\beta(1\rightarrow4)$-linked L-iduronic α-1,3-*N*-acetyl-D-galactosamine disaccharides. Dermatan sulfate isolated from pig skin [9] and umbilical cord [8] has been shown to have a hybrid molecular structure in which glucuronic acid is substituted for iduronic acid in the polymer chain, which renders these linkages susceptible to enzymatic cleavage by hyaluronidase and other $\beta(1\rightarrow4)$-glycosidases. Determination of molecular weights before and after digestion with hyaluronidase therefore probes the polymer for its glucuronic acid substitutions.

Hyaluronidase did not alter the molecular size of dermatan sulfate isolated from hog mucosa. In contrast, treatment with hyaluronidase produced smaller molecular weight fragments of dermatan sulfate from both the young and term human placenta. This indicated that both polymers had glucuronic substitutions. The significantly greater reduction in molecular weight of dermatan sulfate from the young placenta compared with term placenta suggests three possibilities: (*1*) that the polymer from the young placenta contains greater numbers of glucuronic acid substitutions, (*2*) that the substitutions are positioned near the middle of the polymer chain, or (*3*) that the hybrid molecules of each species have the same frequency and pattern of substitutions but there is a far greater proportion of hybrids in the fetal material than exists in the term.

Molecular weight determinations by optical mixing spectroscopy does not permit differentiation of these three possibilities, but it may be possible to do this in principle by characterization of the molecular weight distributions of the samples from line shape analysis [17]. Chemical analysis to resolve this question could not be carried out because of insufficient sample. Limitation in the amount of sample is a frequent problem in structural studies of developing systems. The technique of molecular weight measurements using optical mixing spectroscopy, when coupled with chemical or enzymatic cleavage methods, provides a powerful tool with which to study structural differences in GAG and possibly other types of polymers and proteins in embryonic tissues. The main virtues of the method are that only milligram quantities of sample are needed for analysis and the measurements do not destroy the sample, which can then be utilized for other analytic procedures.

The biologic implications of the compositional and structural changes in GAG found in the placenta at 12–18 weeks and 40 weeks of gestational age remain speculative. Theoretically, the increased proportion of sulfated GAG in the 40-week placenta might be expected to change both the physical and chemical characteristics of the ground substance and thereby alter the transport of molecules passing through it. In terms of steric effects, sulfated polymers would be stiffer than nonsulfated polymers and more extended in configuration, thereby increasing the physical domain which they occupy and also the intramolecular spaces of the gel. This would result in increased efficiency in the exclusion of large macromolecules from the gel, and hence in their more rapid transport by bulk flow between tissue compartments. Macromolecules of intermediate size which can partly penetrate the gel would behave differently. Their passage by bulk flow would be slowed but once in the gel they would filter through it more quickly. Transport of small molecules by these two mechanisms of bulk flow and molecular sieving would not be altered by increased sulfation. On the other hand, the increase in anionic charge would be expected to increase the binding capacity of GAG for cationically charged molecules, regardless of size, retarding their passage through the matrix. The net effects of these physical and chemical changes as the placenta ages might be summarized by decreased transfer of small cationically charged molecules with relatively faster transport of most macromolecules by bulk flow.

Some data has been generated in this laboratory which indicates that dibasic amino acids bind to purified chondroitin 6-sulfate in solution. In addition, recent reports indicate that polylysine and polyarginine are tightly bound to sulfated GAG [10, 11].

These considerations suggest that the molecular structure and/or composition of placental GAG may in part regulate the transport of small and large molecules across the placenta, thereby affecting fetal growth. We are currently testing this possibility by

analyses of placenta obtained from term infants who are small for gestational age.

Summary

Chemical, enzymatic and structural analysis of GAG isolated from the human placenta between 12–18 weeks of gestational age and at term (40 weeks) show age-related differences in composition and structure of these polymers. The young placenta contains increased concentrations of chondroitin, whereas the term placenta shows less unsulfated GAG but higher concentrations of dermatan sulfate and heparan sulfate. Changes in the composition are paralleled by differences in the molecular structure of chondroitin sulfate and dermatan sulfate. The biologic implication of these data may be related to postulated roles of GAG in the regulation of placental transport.

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38. Standard GAG was supplied by Dr. Martin B. Mathews, University of Chicago, Chicago, Illinois, and consisted of hyaluronic acid and chondroitin-6-sulfate from human umbilical cord, chondroitin-4-sulfate from the notochord of rock sturgeon, dermatan sulfate and heparin from hog mucosal tissues, keratan sulfate from bovine corneal tissue, keratan sulfate 2 from human costal cartilage, and heparin sulfate from bovine lung.
39. Chondroitinase AC II was prepared from *Arthrobacter aurescens,* and chondroitinase ABC was prepared from *Proteus vulgaris* by Seikagaku Kogyo Co., Ltd., Tokyo, Japan, and distributed by Miles Laboratories, Inc., Kankakee, Ill.
40. The standards were 6-, 4-, and nonsulfated galactose derivatives of 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranasyluronic acid), prepared by Seikagaku Kogyo Co., Ltd., Tokyo, Japan, and distributed by Miles Laboratories, Inc., Kankakee, Ill.
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