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The Composition of Glycosaminoglycans in Developing Rabbit Lungs (40863)[1]

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Abstract. The components of connective tissue of the lung, fundamentally important to pulmonary function, undergo changes during development of the lung. In an effort to understand connective tissue macromolecular changes, glycosaminoglycans (GAG) were isolated from the lungs of rabbits of various ages, newborn to 24 weeks old. The concentration of total GAG was 15% greater in the lungs of 1-week-old rabbits than in the lungs of newborns. The total GAG concentration gradually decreased between 1 week and 12 weeks of age from 4.2 mg/g dry-defatted tissue to 1.5 mg/g dry-defatted tissue, and then reached a plateau. The predominant GAG in the lungs of newborn and 1-week-old rabbits were hyaluronic acid and chondroitin 4-sulfate. These GAG decreased with age while other GAG, particularly chondroitin 6-sulfate, increased. Analyses of heparan sulfate fractions from lungs of rabbits of various ages showed a microheterogeneity in the molecule. Increase in sulfation of heparan sulfate was observed with age. The observation suggests that not only the total concentration of GAG alters with age but individual GAG with specific physiologic functions also vary with growth and development of the lung.

The connective tissue of the lung is of fundamental importance to pulmonary function. Unlike collagen and elastin which, when combined, comprise more than 90% of the dry weight of the tissue, the proteoglycans in pulmonary tissue are minor components. However, the proteoglycans are important because of their multiple biologic functions in the lung, e.g., their effect on gas–water, fluid, and electrolyte balance (1) anticoagulant activity (2), and influence on connective tissue synthesis. The connective tissue components further provide mechanical support to the organ system (3). Although considerable information is available about the changes of collagen (4) and elastin (5) of the lung with age, little is known about proteoglycans in developing tissue. Horwitz and Crystal (6) studied the content and synthesis of glycosaminoglycans (GAG) in the developing lung of rabbits at four stages: prenatal (−6 days), 7, 45, and 180 days old. They observed that the total GAG was relatively constant from late in gestation through the weanling period, but increased as the animals reached maturity. We explored these changes further and studied GAG composition in the lungs of rabbits in more detail, including the sulfation of heparan sulfate, which is a predominant GAG in the gas-exchange tissue.

Materials and methods. New Zealand white rabbits at various ages, viz. newborn, 1, 2, 4, 8, 12, 16, and 24 weeks old were used as a source of lungs. The animals, five in each age group, were exsanguinated by decapitation and the lungs were dissected free of extraneous tissue. There was no evidence of infection in any of the lungs. The parenchyma, which was free of bronchi and large blood vessels, was finely minced and dry defatted over several changes of acetone at 5° over a period of 10 days.

Isolation of GAG. Glycosaminoglycans were isolated from dry-defatted tissue by procedures previously described (7, 8). Briefly, after an initial treatment with 2% NaOH at 24° for 48 hr, the tissue was repeatedly digested (three times) with papain at 65° for 48 hr in 0.1 *M* phosphate buffer,

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pH 6.5, containing ethylenediaminetetraacetic acid (EDTA) and cysteine HCl. Complete dissolution of the tissue was noted in all samples. An equal volume of 12% trichloroacetic acid (TCA) was added to the digest to precipitate protein material; the latter separated from the GAG solution by centrifugation at 14,000 *g* for 30 min. The sedimented protein material was washed three times with 6% TCA and the washings were added to the original supernatant. The pooled supernatant and washings were exhaustively dialyzed against distilled water to remove TCA and then were lyophilized. The lyophilized GAG material was dissolved in distilled water and uronic acid was determined on a small portion of the solution. The remainder was used for fractionation into individual GAG.

In preliminary experiments the TCA-precipitated protein materials of samples from four different age groups of rabbits were dissolved in 2% NaOH and the solution was neutralized, dialyzed against distilled water, concentrated to a small volume on a steam bath, and analyzed for uronic acid. No detectable uronic acid was noted in these precipitates.

Fractionation of GAG. Fractionation of GAG was achieved on a Dowex-1 Cl^-, 200–400 mesh column, as previously described (9). The GAG from the column were eluted with a $NaCl-MgCl_2$ gradient (0.5–3.0 *M*). The fractions were analyzed by orcinol–H_2SO_4 reaction in a Technicon sugar analyzer. Fractions corresponding to peaks on the recorder chart were collected using a stream splitter in the manifold. These were pooled, dialyzed against distilled water, lyophilized, and analyzed.

Analyses. Hexuronic acid was determined by the Dische method (10) and hexosamine by the method of Boas (11), omitting the use of resin after the sample was hydrolyzed by 4 *N* HCl for 14 hr at 100°. Gas–liquid chromatography procedures were used for differential determination of glucuronic acid and iduronic acid (12) and glucosamine and galactosamine (13), for estimation of *N*-acetyl groups (14) and total sulfate content (15). *N*-Sulfate determination was done by nitrous acid degradation (16). Isomeric chondroitin sulfates obtained from the Dowex-1 column were estimated by the method of Saito *et al.* (17). Electrophoresis of GAG was performed on cellulose acetate strips in pyridine/formate, pH 3.6, or 0.3 *M* cadmium acetate buffer, pH 4.1. GAG were localized by alcian blue stain as described before (18).

Results. Figure 1 illustrates the total GAG concentration of lungs of rabbits of different ages. In this group of rabbits the highest concentration of GAG (mean value 4.2 mg uronic acid/g dry-defatted tissue) in the lungs was noted in 1-week-old rabbits. Although statistically not significant, the mean concentration of GAG at this stage of development showed a 10% increase over the concentration found in the newborns. In 4-week-old rabbits, the GAG concentration decreased to a mean value of 2.0 mg uronic acid/g dry-defatted tissue. Thereafter, the values slowly decreased to about 1.5 mg in 12-week-old rabbits and then remained at that level until 24 weeks of age. The results are somewhat different than those obtained by Horwitz and Crystal (6) who observed a less distinct decrease in the concentration of GAG in the lungs from 7- to 45-day-old rabbits than that we observed from 1- to 8-week-old rabbits. Further, in the group of rabbits we studied the GAG concentrations in the lungs did not change appreciably after 8 weeks, although Horwitz and Crystal (6) noted a 60% increase of GAG in the lungs of 180-day-old rabbits over 45-day-old rabbits. These variations could be due to variations in the strains of animals or more likely in the GAG isolation methodology.

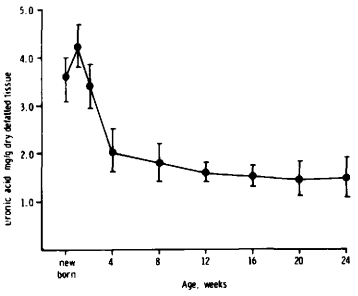

FIG. 1. Glycosaminoglycan concentrations (mean ± SE) expressed as uronate of lung tissues from rabbits at various ages.

Figure 2 illustrates chromatographic profiles of GAG from lungs of 1-, 4-, and 24-week-old rabbits. Differences were noted among the samples in the proportions of specific GAG peaks. A shift toward the right in the position of heparan sulfate, peak II was noted in the GAG from the lungs of older animals. Previous studies (9) showed the areas of these peaks to be proportional to the concentration of GAG. A correction factor of 2.3 is required for the areas of peaks III and IV because of suppression of the oricinol–H_2SO_4 color reaction due to the high salt concentration at which these peaks are eluted from the column. A coefficient of variation of 2% for hyaluronic acid, 4% for heparan sulfate, 10% for chondroitin 4-sulfate, 7% for chondroitin 6-sulfate, 5% for dermatan sulfate, and 6% for heparin was previously noted in the combined chromatographic and enzymatic procedure.

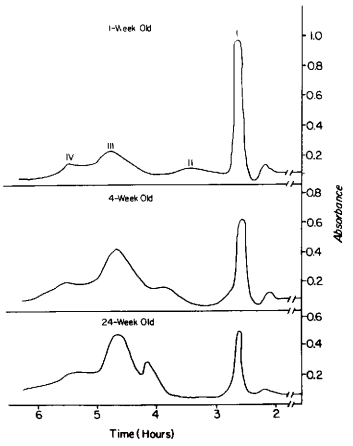

FIG. 2. Dowex-1 Cl^- chromatography profiles of glycosaminoglycans from lung tissues of 1-, 4-, and 24-week-old rabbits. Peak I corresponds to hyaluronic acid, peak II to heparan sulfate, peak III to chondroitin sulfates + dermatan sulfate, and peak IV to heparin.

The composition of individual GAG as percent of total GAG is reported in Table I. In the lungs of newborn and 1-week-old rabbits chondroitin 4-sulfate and hyaluronic acid were the predominant GAG, constituting about 30 and 20%, respectively. Heparan sulfate, chondroitin 6-sulfate, and heparin each represented about 10% of the total GAG and dermatan sulfate composed 15% of the GAG. There was a gradual decrease of the proportions of hyaluronic acid and chondroitin 4-sulfate in the total GAG during the first 8 weeks of life. Later these GAG remained unchanged for the period of study. Chondroitin 6-sulfate, which constituted about 10% of the total GAG in newborn lungs, gradually increased to 30% in the lung tissue of 12-week-old rabbits. Although not marked, there were increases with age in the percent heparan sulfate and dermatan sulfate formed of the total GAG. There was no change with age in the concentration of heparin in the lung tissues, and these results, in general, agree with those of Horwitz and Crystal (6). They differ, however, from their observation of a threefold increase of dermatan sulfate in the lungs of newborn rabbits to weanling rabbits. In this study, we noted only a 25% increase of dermatan sulfate in the lungs of 24-week-old rabbits over that observed in newborns. Horwitz and Crystal (6) did not

TABLE I. THE COMPOSITION OF GAG IN THE LUNGS FROM RABBITS OF VARIOUS AGES

	% of total GAG (mean ± SE)					
	HA[a]	HS	C4-S	C6-S	DS	Hep
Newborn[b]	22.8 ± 3.2	11.7 ± 2.4	30.6 ± 4.0	10.6 ± 1.4	16.1 ± 3.4	8.3 ± 1.2
1 week old	21.0 ± 2.8	11.9 ± 1.8	33.8 ± 5.1	9.1 ± 2.3	15.0 ± 1.8	9.1 ± 2.0
2 week old	16.0 ± 4.1	14.7 ± 2.2	30.0 ± 3.6	15.9 ± 3.4	12.7 ± 2.6	10.9 ± 1.6
4 week old	11.1 ± 3.6	14.0 ± 4.2	24.0 ± 3.2	25.0 ± 4.1	15.0 ± 3.1	11.0 ± 1.5
8 week old	10.0 ± 4.3	17.8 ± 3.8	16.1 ± 2.9	27.8 ± 4.2	18.9 ± 2.4	8.9 ± 2.4
12 week old	11.3 ± 4.0	18.9 ± 4.0	11.8 ± 3.1	31.3 ± 3.8	18.1 ± 2.2	6.8 ± 1.9
16 week old	9.3 ± 3.8	19.3 ± 2.9	10.7 ± 2.6	30.0 ± 3.7	21.3 ± 4.1	10.0 ± 2.6
24 week old	10.2 ± 2.9	17.3 ± 3.6	10.0 ± 2.9	32.0 ± 4.5	20.0 ± 3.6	11.3 ± 3.1

[a] Abbreviations: HA, hyaluronic acid; HS, heparan sulfate; C4-S, chondroitin 4-sulfate; C6-S, chondroitin 6-sulfate; DS, dermatan sulfate; Hep, heparin.

[b] Number of animals in each group is five.

estimate heparin and heparan sulfate separately, so it is possible that the increase of heparin + heparan sulfate with age they reported is due to an increase of heparan sulfate alone. We noted age-related changes in heparan sulfate concentrations but not in heparin concentrations.

The Dowex-1 Cl^- column chromatograms of GAG from individual animals show a difference in the relative position of peak II that corresponds to heparan sulfate. The fractions corresponding to the heparan sulfate peak in the chromatogram were, therefore, extensively analyzed and the results are reported in Table II. Although no difference was noted in the molar ratio of uronic acid to hexosamine between age groups, greater proportions of total sulfate and *N*-sulfate groups to hexosamine were noted in older animals (>12 weeks old). The proportion of *N*-acetyl groups to hexosamine in older animals (>8 weeks) was noted to be less.

Similar analyses of chromatography peaks III and IV would have been interesting but were not performed because of the limited material in peak IV and the heterogeneity of peak III, which contained a mixture of isomeric chondroitin sulfates.

Discussion. The observations made in this study indicate that total GAG, as well as individual GAG, in the lungs of rabbits vary markedly with age. These changes in the interstitial components of the lung are obviously related to a difference in their synthesis by the cells and perhaps activity of different cells comprising parenchyma. The earlier studies of biosynthesis of GAG by Horwitz and Crystal (6) indicate that increases in the concentration of GAG precede the maximum rate of synthesis. Further, the concentrations of individual GAG do not change parallel to one another with age. Presumably, the GAG concentration in the lung during development is controlled by a balance of synthesis and degradation by various mechanisms that may include alterations in the cell population of lung (19). Alveolar types I and II epithelial cells, endothelial cells, and interstitial mesenchymal cells are the major types in the parenchyma. These may be responsible for the synthesis and degradation of GAG, with the exception of heparin, whose synthesis is likely related to mast cells. The individual GAG concentrations in the lung may parallel the alterations in the population of cells as the lung grows.

Lung parenchyma contains all of the known GAG, with the exception of keratan sulfate (6, 8, 20) although the relative proportions of individual GAG vary in different sites or tissues of the lung (8), probably related to the specific physiologic function. The lungs in this study were not dissected into gas-exchange, pleura, and bronchioles as in previous studies (8) with bovine lung, because of practical limitations, particularly dealing with newborn and young rabbit tissue. Yet the observations made in the study are particularly significant. The high proportion of hyaluronic acid observed in the newborn and 1-week-old rabbit lungs may have a specific function in these young

TABLE II. ANALYSES OF HEPARAN SULFATES FROM THE LUNGS FROM RABBITS OF VARIOUS AGES[a]

	Moles/mole hexosamine (mean ± SE)			
	Uronic acid	Total sulfate	*N*-Sulfate	*N*-Acetyl
Newborn[b]	0.84 ± 0.11	0.78 ± 0.08	0.14 ± 0.02	0.79 ± 0.10
1 week old	0.97 ± 0.14	0.76 ± 0.09	0.14 ± 0.02	0.82 ± 0.09
2 week old	1.01 ± 0.09	0.89 ± 0.09	0.16 ± 0.03	0.81 ± 0.08
4 week old	0.98 ± 0.10	0.87 ± 0.10	0.15 ± 0.02	0.83 ± 0.08
8 week old	1.12 ± 0.09	0.97 ± 0.09	0.20 ± 0.04	0.74 ± 0.12
12 week old	0.87 ± 0.08	1.18 ± 0.12	0.18 ± 0.02	0.74 ± 0.11
16 week old	1.18 ± 0.12	1.21 ± 0.11	0.19 ± 0.02	0.72 ± 0.10
24 week old	1.20 ± 0.11	1.19 ± 0.13	0.19 ± 0.04	0.70 ± 0.13

[a] Heparan sulfate was obtained from the fractionation of GAG on Dowex-1 Cl− column as shown in Fig. 1.
[b] Number of animals in each group is five.

animals. Alveolar ventilation in newborn and young animals is greater than in adults (21), and consequently there is need for greater gas–water, fluid, and electrolyte exchange in young animals. Hyaluronic acid may facilitate this function by trapping water in the interstitium. The observed change in the concentration of chondroitin sulfates and dermatan sulfate with age may influence the mechanical functions of the lung through interaction with developing collagen fibers (22).

The marked differences in total sulfate and *N*-sulfate contents and *N*-acetyl groups in heparan sulfates from lungs at the different ages indicate a microheterogeneity of this macromolecule. The results also suggest a relationship between sulfation and growth. A similar relationship was previously observed only for chondroitin sulfates (23) and keratan sulfate (24). The significance of changes in the concentration as well as the composition of heparan sulfate is not clear, but our recent studies (25) of bovine lung suggest that heparan sulfate proteoglycan is the major proteoglycan in the gas-exchange and pleural tissues of bovine lung, it possesses anticoagulant activity, and it inhibits platelet aggregation.

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