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Galactosaminoglycans from normal myometrium and leiomyoma

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Abstract

In many tumors, the amount of chondroitin sulfate in the extracellular matrix has been shown to be elevated when compared to the corresponding normal tissue. Nevertheless, the degree of chondroitin sulfate increase varies widely. In order to investigate a possible correlation between the amount of chondroitin sulfate and tumor size, several individual specimens of human leiomyoma, a benign uterine tumor, were analyzed. The glycosaminoglycans from eight tumors were extracted and compared with those from the respective adjacent normal myometrium. The main glycosaminoglycan found in normal myometrium was dermatan sulfate, with small amounts of chondroitin sulfate and heparan sulfate. In leiomyoma, both dermatan sulfate and chondroitin sulfate were detected and the total amounts of the two galactosaminoglycans was increased in all tumors when compared to normal tissue. In contrast, the heparan sulfate concentration decreased in the tumor. To assess the disaccharide composition of galactosaminoglycans, these compounds were incubated with bacterial chondroitinases AC and ABC. The amounts of L-iduronic acid-containing disaccharides remained constant, whereas the concentration of D-glucuronic acid-containing disaccharides increased from 2 to 10 times in the tumor, indicating that D-glucuronic acid-containing disaccharides are responsible for the elevation in galactosaminoglycan concentration. This increase is positively correlated with tumor size.

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Glycosaminoglycans (GAGs) are heteropolysaccharide chains made up of disaccharide-repeating units, in which one sugar is a hexosamine and the other is either a neutral sugar or a uronic acid. The GAG chains are sulfated to different extents and at different positions. The carboxyl groups of the uronic acids and the sulfate residues give the GAGs a polyanionic nature. With the exception of hyaluronic acid, which is not sulfated and occurs as free chains in tissues, every GAG is covalently linked to a protein, forming proteoglycans.

Proteoglycans are important components of cell surface and extracellular matrices and individual proteoglycans interact specifically with other matrix components, such as collagen, laminin, and fibronectin, as well as with growth factors and cytokines (1-3). These interactions may affect cell growth, migration, adhesiveness, and differentiation, and many of these functions seem to be dependent on the GAG side chains (4). Sampaio and Dietrich (5) have first shown

that the type and quantity of GAGs in mature normal tissues differ from those found during embryonic development and in tumors (6). Chondroitin sulfate and dermatan sulfate, also referred to as galactosaminoglycans because they contain N-acetylgalactosamine as their hexosamine, are the most common GAGs in the extracellular matrix proteoglycans (7). The uronic acid in chondroitin sulfate is always D-glucuronic acid, whereas dermatan sulfate is a hybrid polymer, containing both L-iduronic acid and D-glucuronic acid residues.

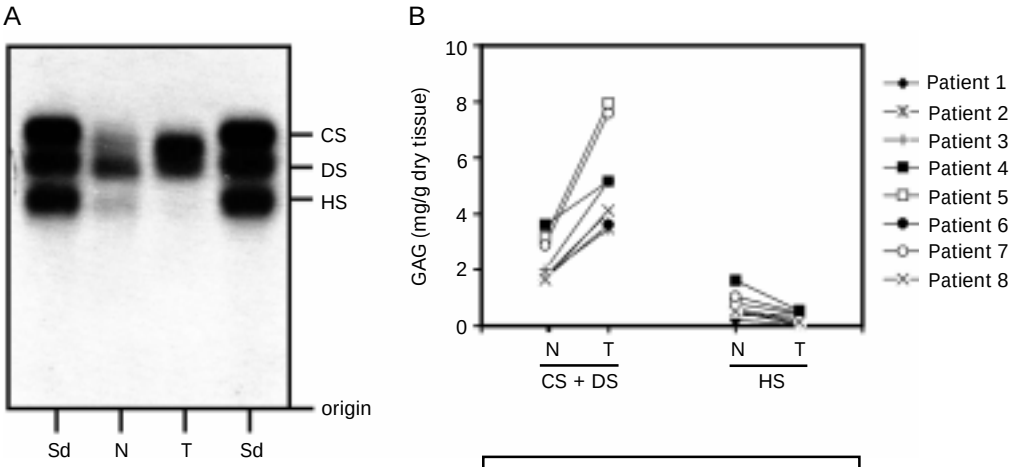
Chondroitin sulfate was greatly increased in transformed cells in culture compared to normal cells (8). The amount of extracellular chondroitin sulfate in many tumors is also high when compared to the normal tissue of origin (6,9), but the magnitude of this increase varies widely, depending on the size and type of tumor (10).

The first study on the composition of sulfated GAGs in the uterus is from the 1960's (11), when it was shown that normal myometrium contains heparan sulfate, chondroitin sulfate, and dermatan sulfate. The proportions among these three GAGs did not vary significantly in pre- and postmenopause or in pre- and postgestation uterus. Although variations in the concentration of GAGs have been found, it was not possible to establish correlations between these variations and any of the analyzed parameters, such as patient age. The only correlations established were a significant decrease of uterine GAG during pregnancy (12,13) and an increase in chondroitin sulfate in leiomyoma, a benign tumor of the myometrium (6), and in leiomyosarcoma, a malignant tumor of the same tissue (14). Again, the magnitude of this increase varied from 2 to 20 times (6).

In order to determine if this variation in chondroitin sulfate concentration is a function of tumor class or size, or is due only to biological variability, several tumors of the same type were individually analyzed. The aim of the present investigation was to perform a more systematic study on the GAG composition of leiomyoma, in order to establish a possible correlation between GAGs and some parameters of neoplastic development in human myometrium.

Human leiomyomas were obtained shortly after surgical excision. Tumoral and adjacent normal myometrium were dissected and stored at -20°C until use. The frozen tissues were weighed and ground in 10 volumes of acetone. After standing overnight at room temperature, the tissues were collected by centrifugation and dried. About 0.45 g of the acetone powder thus obtained from normal myometrium and leiomyoma was incubated overnight with papain (2 mg/ml in 0.06 M phosphate-cysteine buffer, pH 6.5, containing 20 mM EDTA) at 60°C. Afterwards, debris was removed by centrifugation (5,000 *g*) for 15 min. Trichloroacetic acid and NaCl were added to the supernatant up to 10% and 1-M final concentrations, respectively. The mixture was left to stand for 10 min at 4°C and the precipitate formed was removed by centrifugation at 4,000 *g* for 10 min at 4°C. The GAGs were precipitated from the supernatant by the slow addition of 2 volumes of ethanol with shaking. After 18 h at -20°C the precipitate was collected by centrifugation, vacuum dried, resuspended in 0.5 ml of a solution containing deoxyribonuclease I (1 mg/ml) and 50 mM sodium acetate buffer, pH 6.0, and incubated at 30°C for 12 h. The GAGs were analyzed by a combination of agarose gel electrophoresis and degradation with specific bacterial mucopolysaccharidases.

Comparative results for leiomyoma and normal adjacent myometrium obtained from eight patients revealed important structural alterations of GAG content. Figure 1A shows the electrophoretic migration of GAGs extracted from one of these samples. The difference in GAG composition is evident. In normal myometrium, the main sulfated GAG is dermatan sulfate with small amounts of chondroitin sulfate and heparan sulfate,



	µg Disaccharides/g dry tissue	
	HS	CS + DS
N	709 ± 158	2317 ± 270
T	295 ± 70*	5133 ± 615*

Figure 1. Sulfated glycosaminoglycans (GAGs) from normal human myometrium (N) and leiomyoma (T). *A*, Agarose gel electrophoresis of GAGs extracted from leiomyoma and normal adjacent myometrium from patient 1: about 5 µl of the GAGs extracted as described in the text was submitted to agarose gel electrophoresis in 0.05 M 1,3-diaminopropane acetate buffer, pH 9.0, as previously described (6). Sd: mixture of standard GAGs; CS: chondroitin sulfate; DS: dermatan sulfate; HS: heparan sulfate. *B*, Amounts of CS plus DS (CS + DS) and HS extracted from eight tumors and from their respective adjacent normal myometrium. The GAGs were quantified by densitometry (525 nm) of the agarose gel slabs and the figure shows the individual results obtained. Data are reported as the mean ± SEM. *P<0.01 compared to group N (Student *t*-test).

while in leiomyoma the main GAGs are chondroitin sulfate and dermatan sulfate. As chondroitin sulfate and dermatan sulfate migrate very close to each other, it was difficult to determine their individual concentrations. For this reason, the galactosaminoglycans (chondroitin sulfate and dermatan sulfate) were quantified together by densitometry of the agarose gel slabs. The concentration of galactosaminoglycans (expressed as mg per g dry tissue) increased in all samples of tumor tissue as compared to the respective adjacent normal myometrium (Figure 1B). In contrast, the heparan sulfate contents decreased in all cases (Figure 1B). The mean values and standard errors are also shown.

To investigate if this increase was due to both chondroitin sulfate and dermatan sulfate, the disaccharide units that compose these polymers were analyzed. The GAGs were incubated with chondroitinase AC (from *Flavobacterium heparinum*) (15) and chondroitinase ABC (from *Proteus vulgaris*) (16). The degradation products formed were analyzed by paper chromatography stained with alkaline silver nitrate (17) and quantified by densitometry. Both dermatan sulfate and chondroitin sulfate are hybrid polymers. Chondroitin sulfate is composed of 4- and 6-sulfated disaccharide units, all of them containing D-glucuronic acid (18). Dermatan sulfate, on the other hand, contains both D-glucuronic and L-iduronic acid residues (19). The combined action of chondroitinases AC and ABC permits to assess the amounts of these disaccharide units in the polymers. Chondroitinase AC degrades chondroitin sulfate and the D-glucuronic acid-containing regions of dermatan sulfate. L-Iduronic acid-containing regions are not substrates for this enzyme but are degraded by chondroitinase ABC. Figure 2A shows that both 4-sulfated and 6-sulfated disaccharides were formed from normal and tumoral GAGs by chondroitinase AC (D-glucuronic acid) but more 4-sulfated disaccharides were produced by chondroitinase ABC, indicating the presence of L-iduronic acid. Nevertheless, the L-iduronic acid contents of tumors were unaltered when compared to the adjacent normal tissue, in contrast to the D-glucuronic acid contents that increased in all cases (Figure 2A) with magnitudes ranging from 1.7 to 11 times. Furthermore, Figure 2B shows that the amounts of D-glucuronic acid-containing disaccharides were positively correlated

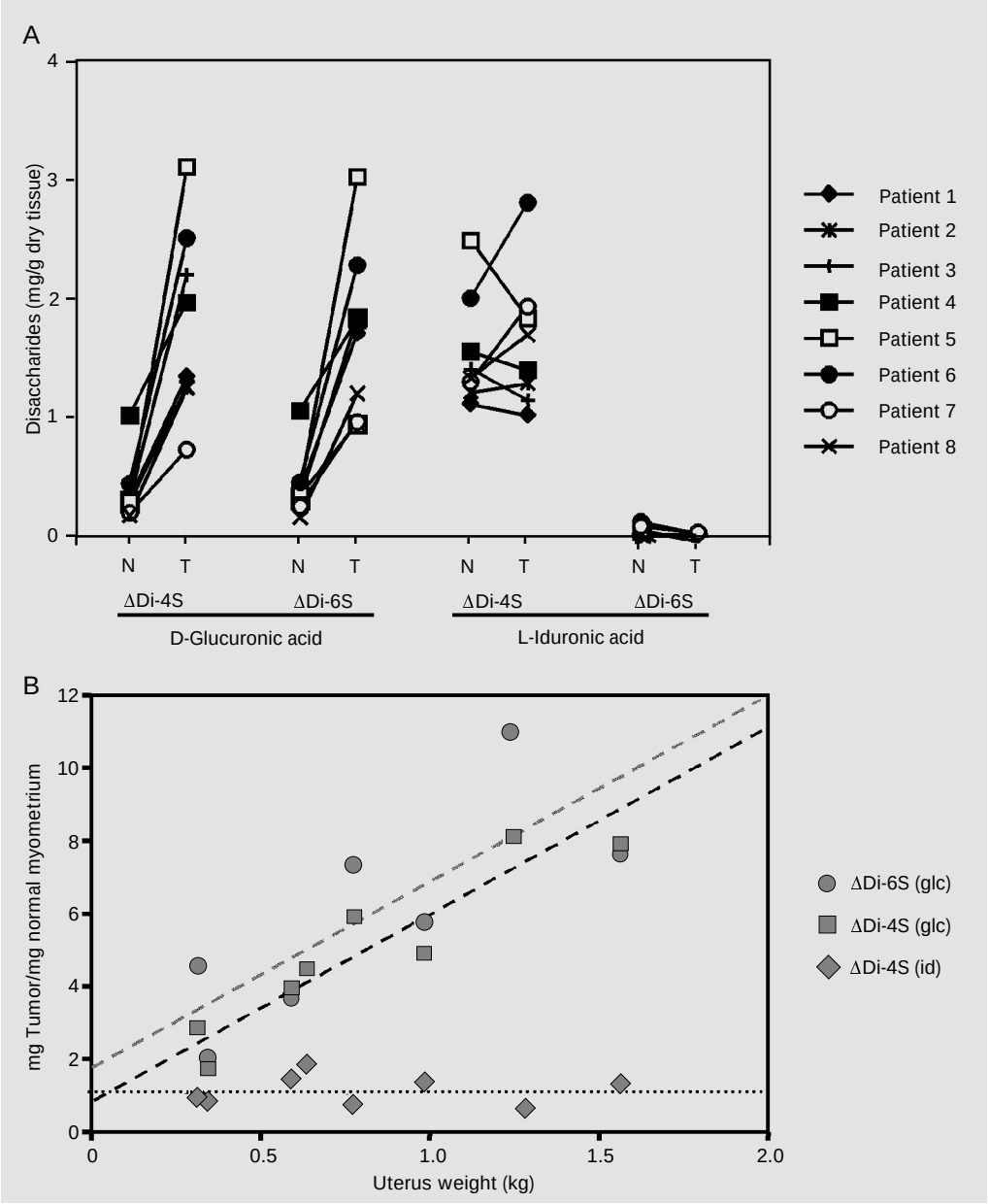


Figure 2. Disaccharide composition of galactosaminoglycan from normal human myometrium (N) and leiomyoma (T). *A*, Concentration of unsaturated disaccharides produced by bacterial chondroitinases AC (D-glucuronic acid) and ABC (L-iduronic acid): incubation mixtures containing 30 µg of glycosaminoglycans and 20 x 10^{-3} U of either *Flavobacterium heparinum* chondroitinase AC or *Proteus vulgaris* chondroitinase ABC were prepared in 0.1 M ethylenediamine-acetate buffer, pH 7, in 30 µl final volumes. After overnight incubation at 37ºC, the mixtures were applied to Whatman No. 1 filter paper and submitted to descending chromatography in isobutyric acid: 1.25 M NH_4OH, 5:3 (v/v). Reducing products were stained with alkaline silver nitrate (17). The disaccharides that contain D-glucuronic acid were estimated by densitometry of the disaccharides produced by the chondroitinase AC and the disaccharides that contain L-iduronic acid were estimated by the difference between the products formed by chondroitinase ABC and AC. ΔDi-4S: Unsaturated 4-sulfated disaccharides; ΔDi-6S: unsaturated 6-sulfated disaccharides. *B*, Correlation between tumor size and concentration of D-glucuronic acid-containing (circles and squares) and L-iduronic acid-containing (lozenges) disaccharide units in galactosaminoglycans. The values are reported as the leiomyoma/normal myometrium ratio. ΔDi-6S (glc): unsaturated 6-sulfated disaccharides produced from D-glucuronic acid-containing disaccharide units; ΔDi-4S (glc): unsaturated 4-sulfated disaccharides produced from D-glucuronic acid-containing disaccharide units; ΔDi-4S (id): unsaturated 4-sulfated disaccharides produced from L-iduronic acid-containing disaccharide units.

with tumor size.

It is possible that the galactosaminoglycan chains, produced in progressively higher amounts as the tumor grows, were not properly processed, possibly due to a lower activity of uronic acid epimerase. This possibility was also raised by Sobue et al. (14), who analyzed benign and malignant tumors of the uterus, and observed that leiomyosarcomas contain considerably larger amounts of chondroitin sulfate than benign tumors, which contain dermatan sulfate with a small proportion of L-iduronic acid.

Elevated matrix chondroitin sulfate has also been correlated with the stage of prostate cancer, and the measurement of chondroitin sulfate concentrations at diagnosis has been proposed to allow stratification of patients with early-stage cancer for different therapies (20).

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